

Restoring faith in anthropology

Rebuttals of a controversial book that alleges malpractice by distinguished anthropologists have so far confused rather than clarified the situation. An independent inquiry is urgently needed.

American anthropologists are going through a traumatic episode that calls for decisive and definitive action. Journalist Patrick Tierney has written a book — *Darkness in El Dorado: How Scientists and Journalists Devastated the Amazon* — that alleges scientific misconduct by high-profile members of the anthropological community. The allegations include experimentation without consent on Yanomami Indians in Venezuela, the possible spreading of measles, fever and other diseases by researchers, and various abuses of indigenous peoples, including a French anthropologist's use of Yanomami boys for sex.

At its annual meeting last month in San Francisco, the executive board of the American Anthropological Association (AAA) announced that a panel would be set up to determine whether there should be a full investigation into the allegations in Tierney's book (see *Nature* **408**, 391; 2000). The seven-member panel was chosen last week. It will deliver its recommendations to the AAA's executive board in February, and the board will then decide whether — and how — to examine allegations about anthropological practices that took place in isolated jungles more than 30 years ago.

But the breadth of the allegations, the difficulty and cost of conducting an international investigation, and the need for a variety of expert perspectives, require a more comprehensive probe if it is to satisfy both US and Venezuelan scientists. Louise Lamphere of the University of New Mexico, the president of the AAA, has already asked the American Association for the Advancement of Science (AAAS) to consider examining the allegations regarding the medical and genetic issues, as these are outside the AAA's normal purview. The AAAS board considered this last week, but has yet to announce its decision.

Given the enormity of the job, the high stakes for anthropology and the personal reputations of those attacked by Tierney, it is imperative that a complete investigation is carried out by an independent agency or commission — possibly with government funding. Such an untainted probe could overcome the partisan politics now playing out in the anthropology community. The difficulty is in identifying an agency with expertise and without bias. There may even be some outside the scientific community who will question whether the AAAS has the necessary independence.

The charges

Tierney's book focuses on the behaviour of anthropologist Napoleon Chagnon and deceased geneticist James Neel, beginning in the late 1960s in isolated villages in Venezuela and continuing into the 1990s. Among other charges, they are accused of collecting blood from the Yanomami for the US Atomic Energy Commission without the natives' consent, and of possibly causing native deaths from using a measles vaccine. Tierney also describes how blood was acquired from the Yanomami with payment in steel machetes or axes. He charges that this was highly damaging to the people's indigenous culture.

The activities alleged by Tierney occurred when Neel and Chagnon were at the University of Michigan, where Neel had started one of the nation's first genetics departments. Chagnon later moved to the University of California at Santa Barbara (UCSB), from which

he retired about a year ago. In statements posted on a UCSB website (<http://www.anth.ucsb.edu>), Chagnon has denied any impropriety.

Early in September, when the book's allegations started spreading in e-mails, supporters of the two researchers jumped into high gear, sending out a barrage of material highly critical of the book. This prompted a series of missteps that have clouded a fair assessment of the book's charges.

For example, invoking guidelines for charges of scientific misconduct, the University of Michigan set up an inquiry panel to examine the charges. On 27 September, the university issued a statement criticizing the book and disputing its central allegations. But Michigan officials did this without ever seeing the book or examining the substantial documentation obtained by Tierney on which the book was based. Later, after receiving an uncorrected proof of the book, Michigan issued an updated document, which itself remains controversial.

Academy statement

Michigan officials also contacted the National Academy of Sciences, which put out a critical statement in early November. This statement (see <http://nationalacademies.org>) has since been challenged by Tierney. Meanwhile, UCSB administrators have undertaken no formal inquiry. An anthropology professor at UCSB has issued a long, critical document. But this document does not represent the official view of the university, although Chagnon's supporters have stated that it does.

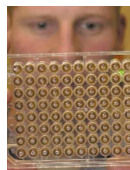
These miscues — which brought groans from the AAA leadership, as it has made its job more difficult — have prompted so much confusion that an independent investigation is imperative.

But the most important need is to restore faith — not only in anthropology but also in medicine — that may have been lost as a result of the book's allegations. Conducting research today in the natural environments of emerging nations is a delicate matter. The rights of host governments, indigenous peoples and various other interests must all be considered, in a political climate starkly different from when the Neel/Chagnon team first trekked into the forest. Furthermore, because Tierney has questioned whether an appropriate measles vaccine was used on the Yanomami in 1968, there are fears that it will now be harder to vaccinate or treat indigenous peoples.

To its credit, the AAA has acknowledged that the book identifies weaknesses in anthropological research techniques. And last month it decided to examine how its research guidelines could be refined to better protect human subjects and their cultures.

Time is of the essence for this process. Already, other scientists — including botanists and ecologists — fear that the offences alleged by Tierney will harm their research efforts in developing nations. If the allegations in Tierney's book aren't appropriately addressed, a range of scientific explorations could be damaged. Tierney's book has been called "anti-science" by some of its critics. Although the investigations have yet to be conducted, there are some signs that Tierney's charges cannot be lightly dismissed. It would certainly be "anti-science" to botch an assessment of the legitimacy of his accusations. ■





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Japan to bypass bureaucracy by reshaping science ministries

David Cyranoski and Robert Triendl, Tokyo

Japanese science policy faces a huge shake-up next year, as the government bids to curtail the influence of its own bureaucrats. On 6 January, two new official bodies will come into operation as part of the most comprehensive reorganization of the Japanese government since 1945.

The Council for Science and Technology Policy (CSTP) will oversee science policy, and the Ministry of Education, Culture, Sports, Science and Technology will be in charge of both education and most of Japan's public research and development funding. The government is set to follow the changes with a shake-up of the national laboratories in April.

Takashi Sasagawa, a wealthy legislator, was named last week as the minister for the CSTP, which supersedes the existing Council for Science and Technology. According to Naoko Okamura, deputy director of the council's secretariat, the CSTP will be responsible for general policy coordination, budget oversight and evaluation.

But some worry that Sasagawa, who has



New brooms: Sasagawa (left) and Machimura (right) will take charge of Japan's science policy.

been embroiled in a tax-evasion scandal, may lack the clout to tackle these issues — and that his post may not survive the next cabinet reshuffle.

In the past, the council was unable to coordinate policy effectively because it was viewed as part the Science and Technology Agency (STA) — one of the agencies competing for funds — which provided it with

administrative support.

It has been suggested that the now-independent council will oversee special programmes, such as last year's Millennium Project (see *Nature* 401, 3; 1999). The council is certain to play an important part in selecting future research initiatives.

Nozomi Sagara, a deputy director of the research planning division at the Agency of Industrial Science and Technology, says that although the council will not supervise agencies or ministries directly, it will set out policies for them to follow.

Akiyoshi Wada, director of the RIKEN Genomic Sciences Center, one of the STA's largest research institutes, thinks that the new arrangement will help to encourage long-term basic research. "Everybody is thinking about tomorrow, but never the day after tomorrow," he says.

The Ministry of Education, Culture, Sports, Science and Technology will be headed by Nobutaka Machimura, a legislator with close ties to the prime minister. The ministry will control about two-thirds of Japan's ¥3.3 trillion (US\$30 billion) annual research and development budget.

There is some concern among former STA officials and in the scientific community that the traditionally conservative education ministry will put the brakes on more adventurous research programmes. STA officials also worry that funds needed to refurbish Japan's ailing national universities — around ¥3 trillion — will be diverted from STA research projects. ■

Germany sets up electronic archive

Quirin Schiermeier, Munich

In response to complaints from researchers about the cost of scientific journals, Germany's largest network of laboratories plans to build a standardized desktop information system for all its scientists.

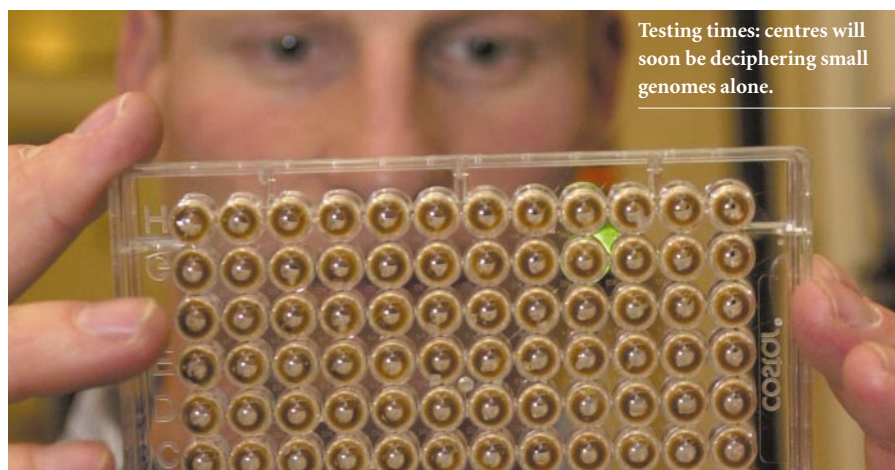
The Max Planck Society (MPS) has created a Centre for Information Management in Garching, near Munich. Its role will be to enable scientists at its 78 laboratories to publish their work in open-access electronic repositories.

When the centre opens next month, its managers will decide whether the MPS should operate its own server, or get involved in similar initiatives elsewhere. These include E-BioSci, a publication server for the life sciences managed by the European Molecular Biology Laboratory, and PubMed Central, a similar project run by the US National Institutes of Health.

But the centre's main partner will initially be the Los Alamos National Laboratory in the United States, whose e-print archives are the primary means of electronic communication in areas such as high-energy physics, maths and computer science. Richard Luce, head of Los Alamos' 'Library Without Walls' project — which provides digital library resources — is advising the new centre.

There is also concern that German scientists have been generally slow to respond to the information revolution. "No one is forging a clear path," says Robert Schlögl, scientific director at the Berlin-based Fritz Haber Institute and a member of the MPS steering group on electronic information. "If we let these developments pass us by, we will end up completely dependent on US technologies." ■

► <http://www.mpg.de/it/cim/director.html>



Testing times: centres will soon be deciphering small genomes alone.

Forces for collaboration falter with human genome in sight

Paul Smaglik, Washington

The cooperation that characterized the international Human Genome Project (HGP) is shifting rapidly towards competition as the project's members vie to decipher the genetic codes of other species.

Leaders of US sequencing centres have described the fragmentation of the consortium as inevitable — especially as the group nears its goal of publishing a draft version of the human genome.

But other researchers wonder if the consortium is breaking up prematurely. They believe a historic opportunity has been lost to build and maintain a distinctively collaborative approach to genomics.

Most of the human genome was sequenced at four centres in the United States and one in the United Kingdom. But many other universities and centres in America, Europe and Japan also took part. The links between the partners have been breaking up more rapidly than some of them had hoped.

Ironically, one of the things driving the sequencers apart is the whole-genome shotgun sequencing technique that once brought them together. The announcement in 1998 by Celera Genomics, based in Rockville, Maryland, that it would use the technique to sequence the human genome before the HGP, helped to galvanize the publicly funded consortium.

Whole-genome shotgun sequencing lends itself to a centralized approach, as any facility with enough sequencers can break the genome into millions of pieces, read them and reassemble them. In contrast, the HGP divided the genome into small segments, assembling the pieces soon after they were read.

The US National Institutes of Health (NIH) tacitly acknowledged the merits of Celera's approach when it revised its plans to sequence the mouse. Initially, the NIH considered applying the same clone-by-clone



Gene shift: Lander says partners will compare notes, not share projects.

technique used by the HGP. But the National Human Genome Research Institute has decided instead to sequence most of the mouse genome by the shotgun method, using the clone-by-clone approach only to build a map to help piece the shotgun data together.

The validation of Celera's technique — and the build-up in the number of sequencing machines at each major centre to prevent Celera from taking the lead — means that individual centres will soon be able to decipher small genomes on their own. The UK Wellcome Trust's Sanger Centre, near Cambridge, is taking on the zebrafish genome alone (see *Nature* **408**, 503; 2000), and the US Department of Energy's Joint Genome Institute in California sequenced 15 bacterial genomes in an October "Microbial Marathon" that also served, in effect, as a declaration of independence.

Richard Gibbs, director of the sequencing centre at Baylor College of Medicine, Houston, says that the loosening of the consortium was inevitable. But he thinks this is happening too quickly. "It makes most sense to enjoy the fruits of its efficiency as long as possible," he says.

Bob Waterston, however, director of the sequencing centre at Washington University in St Louis, says that working alone makes projects easier to manage.

Eric Lander, director of the Whitehead Institute at the Massachusetts Institute of Technology, does not foresee the consortium disbanding completely. Instead, he suggests it will move from sharing projects to discussing the merits of different sequencing strategies and technologies.

Faster sequencing slows down release of mouse gene data

The whole-genome shotgun technique that has been adopted by members of the Human Genome Project (HGP) is affecting the consortium's ability to follow through on its promises over data release.

Before the high-throughput technique was taken up by the project, consortium members had agreed to place new data on a public database every 24 hours. HGP members had used this standard to differentiate the public project from the private one run by Celera Genomics of Rockville, Maryland. Celera intended to release its human data only on publication.

But the publicly funded centres are now using the shotgun technique to sequence the mouse and other organisms. And they are finding that this makes it difficult for them to stick to their agreement to release sequencing data immediately.

The HGP's initial sequencing methods lent themselves well to daily data release, because scientists could continually combine small fragments of deciphered DNA into larger ones. But shotgun sequencing is more problematic, as the technique requires that the small sequence traces produced daily are not assembled until relatively late in the procedure. And the small pieces are shorter than the HGP's agreement specifies for sequence release.

There is no agreement yet on how to deal with shotgun data. Baylor College of Medicine at Houston, one of five sequencing centres that worked originally on the mouse genome, has placed its own small amount of shotgun data on its website. But the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, is not releasing data on its web page. And the institute — along with Washington University in St Louis and the United Kingdom's Sanger Centre, near Cambridge — is sequencing the bulk of the mouse genome.

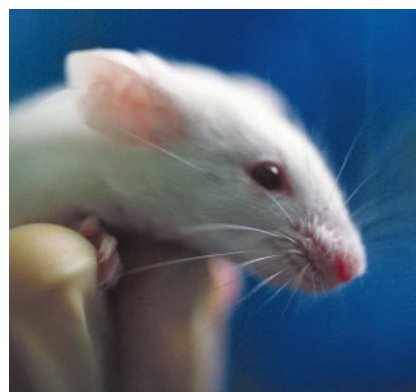
The Whitehead has instead given its data to repositories in Europe and the United States. But these repositories cannot yet accommodate shotgun data. The sequenced mouse fragments have therefore not been made public, even though the total genome of the animal has been sequenced once over.

The data should be available in about three weeks, once programmers have ironed out the technical kinks in the new databases, says David Lipman, director of the National Institutes of Health's National Center for Biotechnology Information.

The public project's switch to shotgun

sequencing will nonetheless require some changes to the HGP's original agreement, says Mark Guyer, assistant director for scientific coordination at the National Institutes of Health's National Human Genome Research Institute, which is managing the US end of the publicly funded mouse project.

Strict adherence to earlier agreements would have meant that the mouse data were not publicly available until the organism was sequenced three times over and then assembled — a milestone targeted for April. Setting up a database of mouse sequence fragments may be an interim solution. "If we had kept to our earlier policy, the data would not have been released for six months," says Guyer. **P.S.**



Shotgun success: the mouse genome has already been sequenced once over.

Publication deal for Celera sparks row over data access

Science magazine has released details of the terms under which it plans to publish Celera Genomics' paper on the human genome — and drawn sharp criticism over the limits that the terms set on data access.

Celera and *Science* have agreed that publicly funded scientists can download up to one megabase of data without signing a material transfer agreement. But privately funded users must sign such an agreement stating that they will not commercialize their results or redistribute the data.

The arrangement has potentially profound significance, some researchers say, because it could set a precedent for scientists to publish papers without unencumbered access to supporting data.

"What will happen if someone else from the academic sector says, 'I have an interesting result to report, but I can't give you all the data?'" asks Harold Varmus, president of the Memorial Sloan-Kettering Cancer Center and former National Institutes of Health director. Varmus was one of several scientists who advised *Science* on the terms. But the advisers are not all in agreement with the announced policy, he says.

When the agreement was announced, Bruce Alberts, president of the National Academy of Sciences, issued a brief statement of support, saying that it may serve as a



Bruce Alberts: deal won't work for private sector.

way to prompt more companies to make their genomic data available.

But this week, Alberts — who says that the statement reflected his own views, not those of the academy — backed off from this. In an interview, he said that the deal could work for publicly funded scientists, but that the terms for private ones appear unworkable. "The data

should be available both to the public sector and private," he says.

The agreement has drawn vocal criticism from some researchers. Ewan Birney, team leader of genomic annotation with the European Bioinformatics Institute in the UK, co-authored an open letter to bioinformaticians attacking the arrangement. He says it is problematic for computational biologists, because they work with large data sets, whose transfer is restricted by the agreement. "This deal is bad for bioinformatics, but palatable for single gene biology," Birney says. **P.S.**

► <http://www.sciencemag.org/feature/data/announcement/genomesequenceplan.shi>

Japan pins hopes on fast-breeder nuclear option

David Cyranoski, Tokyo.

Bucking the international trend towards ending programmes to build nuclear power plants that breed their own fuel, Japan is drawing up plans to reopen its prototype fast-breeder reactor.

The Monju reactor at Fukui, 500 kilometres southwest of Tokyo, has been closed since an accident in 1995, when sodium coolant leaked from a cracked pipe and burst into flames. There were no injuries, but local residents were angered by an attempt to cover up the incident.

Fast-breeder reactors can potentially use uranium fuel many times more efficiently than conventional reactors. But they are expensive to build, and only cover their costs if uranium is expensive, which it has not been. The United States, Britain, France and Germany have all halted their fast-breeder programmes in recent years.

The Monju reactor's reopening is part of a long-term plan released by the Japan Atomic Energy Commission last month.

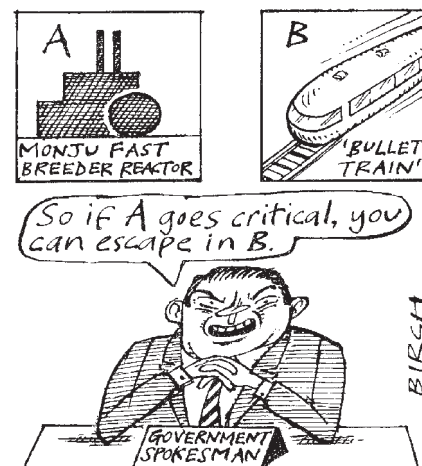
Japanese officials argue that, because of its lack of fossil fuels and other natural resources, the country has no choice but to develop nuclear energy.

The Japan Nuclear Cycle Development Institute (JNC), which operates Monju, must gain approval from the local governor before it can prepare for the reopening. Officials say operations will resume in 2003 at the earliest and run for 20 years.

Their goal is to show that fast-breeder reactors can operate continuously and reliably, and to establish safe and effective techniques for handling sodium. The JNC says it plans to turn Monju into "an international centre for cooperation, open to researchers from Japan and abroad".

But not everyone is convinced that the JNC is adequately concerned about safety at the reactor. Critics complain about the absence of a properly independent safety commission, akin to the US Nuclear Regulatory Commission.

The central government is also



negotiating the possibility of passing a bullet train through Fukui, where Monju is located, arousing suspicions that the government is trying to buy off local criticism. ■

► http://sta-atm.jst.go.jp/jicst/NC/tyoki/siryo/tyoki_e1208/siryo.htm

California invests \$300 million in high tech

Rex Dalton, San Diego

California is pumping \$300 million into three new centres for biomedicine, nanotechnology and telecommunications at the University of California (UC). The centres,

which will be based in San Francisco, Los Angeles and San Diego, will each involve several UC campuses, corporations and investors in an ambitious plan to create new technologies.

A number of state governments, including California, give limited funds to research, but the great bulk of US research funding comes from the federal government. This year, for example, California will receive an estimated \$14 billion in such funding.

But the size of the state initiative — made possible by California's booming economy and budget surplus — may open a new era of major scientific investment by states.

Calling the project "the most ambitious scientific research initiative ever undertaken" by the state, Gray Davis, California's Democrat governor, says the hope is to create "three world-class research and innovation centres with a single mission: invent the future".

The centres and collaborators are: the California Institute for Bioengineering, Biotechnology and Quantitative Biomedicine at UC San Francisco, with UC Berkeley and UC Santa Cruz; the California Nano-Systems Institute at UC Los Angeles, with UC Santa Barbara; and the California Institute for Telecommunications and Information Technology at UC San Diego, with UC Irvine. Each will include new buildings to house laboratories, academics and students.

The centres were recommended by a five-member panel of scientific authorities,

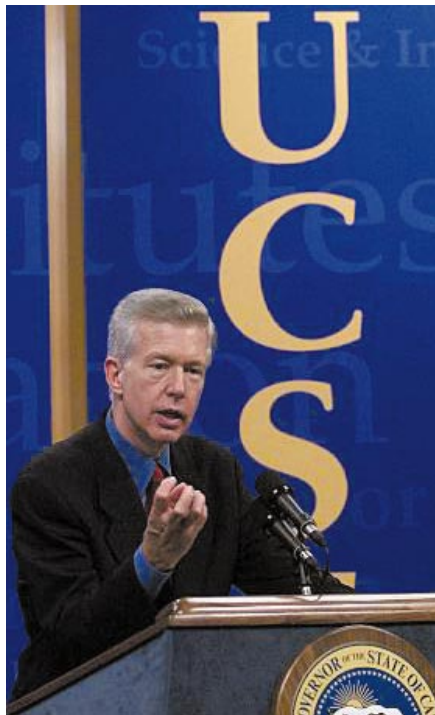
chaired by Richard Lerner, president of the Scripps Research Institute in La Jolla, California. The state rejected three other proposals, but one of these — a centre for information technology at UC Berkeley, to work with three other campuses — was sufficiently strong that UC Davis will seek funding for it in the state legislature's upcoming budget.

A UC Irvine proposal for a centre for systems biology, and a centre for agricultural genomics involving UC Davis and UC Riverside, were not funded. Proponents of the agricultural genomics project were disappointed, but said that promises of industry funding mean that they aim to go ahead with the centre in a modified form.

Under the state's plan, each of the three designated centres will receive \$100 million of taxpayers' money over the next four years. Each centre must also raise twice this from other sources, making the total potential investment worth \$900 million.

Although there are many joint research projects between UC researchers and industry, the explicit mission of these centres to work towards applied technologies has raised some questions about their academic independence.

"It is a challenge to sort how we will do this and preserve the mission of the university," says Zach Hall, vice-chancellor for research at UC San Francisco. "But we are taking on that challenge and want to make it work."



Forward thinking: state governor Gray Davis reveals plans for California to 'invent the future'.

Pesticides implicated in declining frog numbers

Jessa Netting, Washington

Drifting agricultural pesticides may be eroding once-healthy frog populations in the pristine mountain areas of California, US government scientists say.

Researchers from the US Geological Survey (USGS) and the US Department of Agriculture have found that pesticides used by Californian farmers can disrupt an enzyme that regulates the nervous system of frogs in the Sierra Nevada mountains, downwind of farming regions. These same areas are those hardest hit by amphibian losses.

But this is not the whole story of global amphibian decline, according to researchers who met last week in Washington to discuss the issue. The meeting was organized by the biological division of the USGS.

"I think that one thing everyone can agree on is that there is no single cause. There are many interactions," says Harvard University biologist James Hanken.

Amphibians, with their moist, sensitive skins, unprotected eggs and semi-aquatic lifestyle, have long been viewed as biological

indicators of environmental health.

Biologists became aware of a problem in the 1980s, after reports began to accumulate of dwindling or lost frog populations and unusual deformities in amphibians. Evidence came to light that ultraviolet-B radiation, an iridovirus, a chytrid fungus and fluke parasites could each damage amphibian populations. And a study released last April showed that one frog species disappeared when lakes in the California Sierras were stocked with non-native trout.

Another study showed that tadpoles carry much higher loads of parasites in the presence of predatory fish. This indicates that the effects of separate factors can be compounded when they are combined.

Large numbers of frogs with skeletal abnormalities such as missing or extra limbs have also been seen in some populations over the past ten years. Researchers first blamed the deformities on a pollutant — retinoic acid — but later found that extra limbs could be caused by parasites.

The increase in abnormalities may



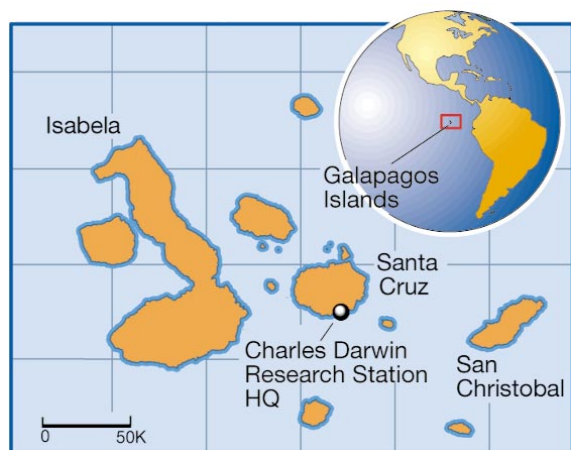
It's no croak: a combination of environmental factors is behind frog deformities and deaths.

indicate an environmental imbalance. But Carol Meteyer, a speaker at the meeting and a USGS veterinary pathologist, said that such malformations did not themselves contribute to the decline of the species they affect.

Despite the uncertainty, USGS biologist Gary Fellers is confident that pesticides are damaging the Californian frogs. He predicts that firm evidence confirming his theory will emerge within two or three years.

♦ http://www.usgs.gov/amphibian_images.html

♦ <http://www.frogweb.gov/tadd/publications.html>



Fishermen on the Galapagos threatened visitors, and ransacked one scientist's home.

Galapagos ecologists under threat from violent protests

Mark Schrope

Forced to flee by angry fishermen protesting against lobster quotas, staff at the Charles Darwin Research Station in the Galapagos Islands spent a night late last month cowering in a mangrove thicket, until they were rescued by the Ecuadorian navy.

Work at the famous ecological research station is returning to normal, but eminent researchers associated with it are calling on Ecuador, which governs the Galapagos, to provide better protection for researchers and to stand by the measures needed to conserve the islands' ecosystems.

Researchers say that during unrest in November, the fishermen made death threats, ransacked one scientist's home, threatened tourists, and forced research and national-park facilities on three islands to close.

The United Nations has made the islands, which lie 1,000 kilometres west of Ecuador, a World Heritage Site. In the 165 years since Charles Darwin's famous visit there aboard the *Beagle*, they have been seen as an exceptional natural evolutionary laboratory, on account of their isolation.

But efforts to conserve their ecosystems have long been at odds with local interests, including those of fishermen. The recent unrest was the latest in a string of similar incidents.

The research station's main facilities are on Santa Cruz Island, and it is operated by the international Darwin Foundation under a charter from the United Nations Educational, Scientific and Cultural Organization. Because it works with park officials to implement and oversee conservation on the Galapagos, the research station has become a focal point of the sometimes violent protests.

But researchers say that the latest incident included bolder and broader attacks on individual visitors. "What's new about this is that

it's targets other than institutions. In that sense it's an escalation," says David Anderson, a biologist at Wake Forest University in North Carolina, who recently returned there from the Galapagos.

Anderson and colleagues at Princeton, and Yale universities and the German Max Planck Institute, representing several projects, say that the violence — in particular the harassment of tourists — raises questions about field researchers' safety and puts conservation efforts in jeopardy.

The attackers, who the researchers say represent only a small fraction of Galapagos fishermen, were demanding that a 50-tonne quota on lobster be increased. The fishing community had taken part in setting the quota, and had agreed to it, but some fishermen became agitated when it was reached just two months into a season that normally lasts four months.

To appease the fishermen, Ecuador raised the quota by 30 tonnes — alarming researchers and conservationists. Fishermen have resisted monitoring since the unrest, and it is not clear whether the limit will stop when the new limit is met.

The protesting fishermen also called on the government to open the Galapagos to shark fishing and long-line fishing for tuna. Conservationists believe that these would be disastrous for the islands' ecosystem.

"Obviously, if violence is rewarded in that way it tends to lead to more violence," says Alan Tye, who was acting director of the research station during the recent trouble. He says at least two of the faction's leaders are in custody, and there are warrants out for others.

But in the past, similar prosecutions have been unsuccessful. Tye says that he hopes that the area will have a stronger police presence. "If all goes well this time it may turn back this process of rewarding violence," he says.

Virtual laboratory will recreate classic biology experiments

Vera Bettenworth and Alison Abbott, Munich

Science historians and computer scientists in Berlin are working to create a virtual nineteenth-century physiology laboratory on the Internet.

The project will allow people to learn how biologists first moved from observation to experiment. Its backers hope that it will give students and the public an insight into the history of science and a stronger understanding of the scientific method.

The virtual laboratory will recreate classic experiments — such as the twitching frog's leg that showed that electrical stimulation can make muscles contract — on the web.

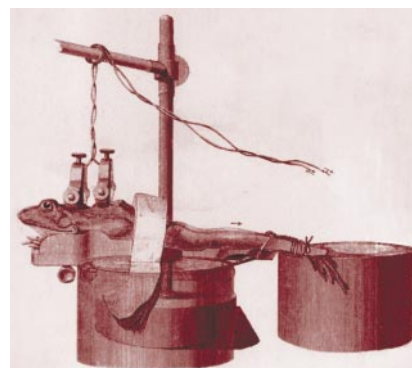
"An experimental approach to life was very new in the nineteenth century," says Hans-Jörg Rheinberger, director of the Max Planck Institute for the History of Science in Berlin, who is coordinating the project.

Rheinberger's team is collecting and digitizing the scattered documents and drawings of early physiologists for the website, which will be launched next year.

Eventually the virtual laboratory will be interactive. "The site will be used not only by archivists and researchers in museums and scientific institutes, but also by the general public," says Sven Dierig, a member of Rheinberger's team. "Historic instruments help the public understand experiments much more easily than today's 'black boxes'," he says.

Robert Bud, head of information and research at the Science Museum in London says that it "is a very ambitious and challenging project which links the public to the scientific community".

But the project needs to raise money for its software. So far, the project has a sponsor, the Volkswagen Foundation, only for its scientific component.



Window on the past: the electrical stimulation of frog muscle will be recreated online.

UN brokers treaty banning persistent organic pollutants

Washington A class of widely used and highly persistent chemical pollutants is to be banned by 122 countries under an international treaty agreed to in Johannesburg last week.

In a meeting organized by the United Nations Environment Programme, diplomats established measures to control persistent organic pollutants, commonly known as POPs. The legally binding contract will regulate the production, shipping, disposal and use of eight pesticides, two industrial chemicals, and two industrial or combustion by-products.

Exempted from the immediate ban are DDT, still heavily used in the developing world to control malaria mosquitoes, and polychlorinated biphenyls (PCBs), which make up parts of old equipment still in use. Use of these two chemicals will be phased out over the next five years.

Participating governments will formally adopt and sign the treaty at a diplomatic conference in Stockholm next May.

Max Planck Society asks for promises of good practice

Munich Germany's Max Planck Society has announced that scientists at its 78 institutes will have to sign an agreement to abide by new guidelines for good practice.

The rules require researchers to archive their data in an accessible form for ten years and to describe experimental protocols in enough detail for them to be reproduced. They disallow honorary authorship on research papers, and require that young scientists are properly supported and supervised.

The society hopes that the guidelines will help to prevent scientific fraud, several cases of which have recently shaken the German scientific community (see *Nature* 398, 13–17; 1999).

International deal to phase out LD50 test

London The LD50 (lethal dose to 50%) test, which has been criticized for its killing of research animals, is to be abolished.

The test, which requires at least 20 rats to receive escalating doses of a trial chemical until half die, will be phased out over the next year. The test is used to assess the potential hazards of chemicals to humans and mammals from accidental exposure.

The United States and Japan originally opposed a ban, but they finally agreed to three alternative tests that reduce the number of animal deaths.

The agreement was announced last week



Death rodent: the United States and Japan have agreed to new chemical tests that kill fewer rats.

by the Organisation for Economic Cooperation and Development.

European Commission speeds up grant decisions

Brussels Researchers applying for European Commission grants will learn of their fate more quickly, following last week's approval of new rules for processing grants.

Currently, applicants are sometimes kept in suspense for months, because European Commissioners are required to rubber-stamp the decisions on projects.

But the commission will now allow researchers to be informed much more quickly. Achilles Mitsos, director-general of the research directorate, will have the power to inform them immediately of the results of their projects' peer review.

White House sets misconduct rules

Washington The White House last week issued requirements for addressing scientific misconduct cases. Agencies have one year to come into compliance with them.

After years of debate, the Office of Science and Technology Policy has finalized a refined definition of misconduct, new data requirements, and guidelines for enforcement. The guidelines are intended to harmonize the procedures used for investigating misconduct at federal research agencies.

♦ www.ostp.gov

Last-ditch meeting fails to bridge climate divide

London Negotiators from Europe and the United States met in Ottawa last week in a last-ditch bid to close the gap that had separated them at the negotiations on climate change last month in The Hague (see *Nature* 408, 503–504; 2000). But they failed to do so.

The meeting, which was also attended by representatives from Canada and Japan, had attempted to reconcile the US demand to be allowed to include agricultural practices when assessing its carbon emission with the European Union's argument that this would reduce the pressure to cut carbon emissions.

A spokesman for the British government says that "some technical issues" were clarified at the Ottawa talks, but no substantial progress was reported. European environment ministers will meet in Brussels next week to discuss their strategy on reaching an accord with the United States.

Leibniz prizes for German biologists

Munich Germany's main university research agency, the DFG, has announced the winners of the 2001 Leibniz Prize, the most important scientific award in Germany.

The seven prizewinners include molecular biologist Eduard Hurt, of the University of Heidelberg, who studies protein structures, and Arthur Konnerth, a neurophysiologist at the University of Munich, who specializes in nerve pathways that regulate mammalian motor systems.

Martin Krönke, an immunologist and cell biologist of the University of Cologne, won for his identification of genes involved in the production of cells that are part of the human immune response. Each winner receives DM3 million (US\$1.4 million), to be spent on their research over the next five years.

Patent regimes should 'take account of poor'

London The British government has proposed an international commission on intellectual property rights to consider how they can be designed "to take greater account of the interests of developing countries and poor people". Topics to be addressed by the commission would include access to genetic resources and "traditional knowledge".

In a white paper (policy document) published this week, the UK Department for International Development says that it is open to "constructive suggestions" on improving the World Trade Organization's agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS).

Universities target bread mould genome

San Diego Four US universities have won a \$5.25 million grant from the National Science Foundation to sequence the genome of the fungus *Neurospora crassa*, which they say will be a valuable model for human genetics.

Full sequencing of the 10,000 to 15,000 genes of the fungus, a type of bread mould, is to be completed over two years. The project involves scientists at the Whitehead Institute in Massachusetts, the Oregon Graduate Institute of Science and Technology, the University of Kentucky, and the Fungal Genetics Stock Center at Kansas University. Researchers at Oregon University will provide the fungal DNA for sequencing.



Swimming against the tide

Toshio Yanagida rejects the conventional biophysical explanation of muscle contraction. No one doubts his technical genius, but could the debate he started ultimately hold back the field? David Cyranoski investigates.

Iconoclast, radical, technical wizard — these are just some of the descriptions that have been applied to Toshio Yanagida, host of last month's Frontiers in Molecular Motors Research symposium in Japan.

Yanagida's technological brilliance has been crucial in developing methods to study what drives muscle contraction. But for 15 years, he has been at odds with the majority of muscle biophysicists over the precise mechanisms involved.

Held on the island of Awaji, just across the water from Yanagida's laboratories in Osaka, last month's meeting was a lively affair. Both sides of the debate presented their latest results. And these served to underline the field's impressive achievements in manipulating and visualizing the individual protein molecules involved in muscle movement.

But from similar experiments, the participants drew very different conclusions. And this continuing trend for both camps to bolster their arguments without finding common ground leaves many in the field perplexed. The debate is where it was ten years ago, says Clive Bagshaw, a biophysicist at the University of Leicester. "It's like nothing has changed."

The argument centres on two proteins:

myosin II and actin. Myosin II has two 'heads' joined by 'neck' regions to a coiled 'tail'. In muscle cells, the tails of myosin II molecules clump together to form 'thick filaments' with the necks and heads jutting out from the sides. Actin, on the other hand, forms helical 'thin filaments', which line up alongside their myosin counterparts. Muscles contract when the thick and thin filaments slide past one another. The puzzle for biophysicists is how the myosin II heads interact with the actin filaments to bring about this movement.

Head start

Most researchers back the lever-arm theory. In this, myosin II heads latch onto a nearby actin thin filament. By flipping in a lever-like motion, each head propels the actin in a 'power stroke' (see figure, opposite). The myosin head then lets go of the actin, the neck cocks back, and the head reattaches at a new point on the thin filament, starting the process again.

Muscle contraction is driven by adenosine triphosphate (ATP), the energy 'currency' of the cell, which binds to the myosin head. In the lever-arm model, a single cycle of actin attachment and power stroke for one myosin

II head involves the hydrolysis of one ATP molecule. Because of this straightforward relationship between energy input and mechanical action, the lever-arm theory is also called the tight-coupling model.

Yanagida proposes that, for each ATP molecule consumed, a myosin II head moves several steps, bumping along the actin filament like a railway wagon rattling down a rickety track. But the details of his theory are frustratingly diffuse. "My opponents are always saying: 'give more interpretation of your results,'" says Yanagida, "but I think it's better not to."

An input of ATP is still crucial, although its precise role in this 'loose-coupling' model is unclear. One possibility, says Yanagida, is that myosin somehow absorbs the energy from ATP hydrolysis, releasing this energy over a series of steps to drive its movement. But the role of ATP might simply be to cause a change in myosin's shape that allows movement to begin. In this case, much of the energy needed for myosin to move against actin would come from the Brownian motion of the molecules in the surrounding fluid. Brownian motion is the random movement of molecules, driven by thermal forces and characterized by collisions between the

molecules. Yanagida believes that the structures of actin and myosin might bias this motion in one direction — although he does not have a full explanation of how this could work.

Seeds of doubt

Yanagida made his mark in 1984, when his group was the first to image a single actin filament in solution¹. But it was in the following year that he sowed the seeds of the debate that continues to this day. By relating the 'sliding velocity' of an actin filament to the consumption of ATP, Yanagida estimated that a single molecule of ATP causes a myosin head to advance some 60 nanometres down an actin filament². This posed a problem for the lever-arm theory, as the dimensions of the myosin head and neck mean that a single ATP molecule should cause a displacement of only around 6 nm. Japanese newspapers immediately hailed Yanagida as a scientific folk hero, claiming that he had overturned a popular 'Western' theory.

Yanagida was working with his mentor Fumio Oosawa at Osaka University, even though Oosawa could only employ him as a technician. After Oosawa retired, the university took the unusual step in 1988 of promoting Yanagida straight to full professor. He has since enjoyed high levels of government funding. His current grant, for his Single Molecule Processes project, is worth more than US\$9.3 million over five years to 2002.

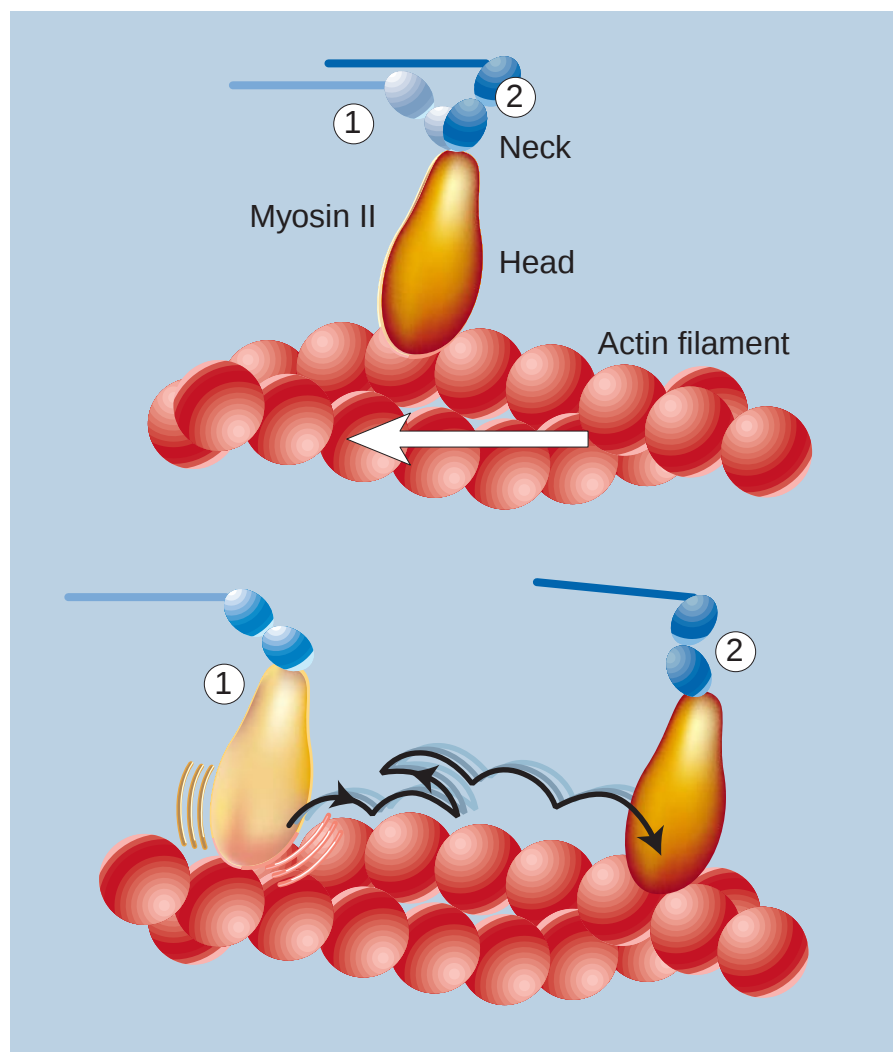
With this generous financial support, Yanagida has assembled a team of 15 scientists in a renovated warehouse. Most have a strong physics or engineering background, and have built their own equipment more or less from scratch. Their technical accomplishments, it is widely agreed by their peers, represent a *tour de force*.

Image is everything

In the 1980s, biophysicists realized that solving the mysteries of muscle contraction would require better technology. They wanted to trap and manipulate individual protein molecules tagged with fluorescent markers. And they needed sophisticated microscopes to watch these proteins interact.

Yanagida helped to overcome many of these hurdles, building the necessary tools in his lab — and, in doing so, spurred others to higher levels of technological sophistication. "He has pushed the envelope," says Ron Vale, a biophysicist at the University of California at San Francisco.

In 1995, for instance, Yanagida's group revealed the first images of a single myosin II molecule binding to an ATP molecule in an aqueous solution³. Imaging fluorescently tagged proteins in solution using conventional microscopes is difficult because the laser that makes the molecules visible also causes scattering and luminescence throughout the solution. This problem was solved by



Flipping neck: the motion of actin and myosin II filaments relative to each other causes muscle contraction. In the lever-arm theory (top), myosin heads repeatedly attach to the actin fibre and then flip forward, propelling the actin along. But according to Yanagida's loose-coupling theory (bottom), the myosin runs along the actin fibre like a railway wagon on a rickety track.

a protégé of Yanagida's, Takashi Funatsu. He refined a technique called total internal reflection fluorescence so that he could limit to 150 nm the depth to which the laser's light penetrated the solution. This set up an 'evanescent' light field, which illuminated the target molecules but reduced the background luminescence more than 2,000-fold.

Other experiments had a more direct bearing on Yanagida's loose-coupling theory. In 1998, Yanagida and his colleagues reported that they had simultaneously observed the binding of ATP and recorded the displacement of a single myosin molecule along an actin filament⁴. The experiment combined Funatsu's evanescent field with another key tool — 'optical tweezers'. These were first applied to muscle biophysics by researchers working in the lab of James Spudich at Stanford University in California⁵. By fixing microscopic beads to either end of an actin filament, the filament can be held taut by directing laser beams — the tweezers — at the beads. From his experiments, Yanagida con-

cluded that the motion of a myosin head down an actin filament induced by one ATP molecule did not occur immediately. He observed a delay of several tenths of a second between the release of adenosine diphosphate, the product of ATP hydrolysis, and the myosin's motion. This delay is incompatible with the tight-coupling model.

Last year, Yanagida and his colleagues refined their estimate of 60 nm for the myosin displacement induced by a single ATP molecule to between 11 and 30 nm (ref. 6). This revision resulted from measurements taken during experiments in which individual myosin II heads were attached to glass microneedles. By measuring the deflection of the needles and their stiffness, the researchers recorded both the displacement of the myosin heads and whether or not they were attached to the actin filament.

They concluded that a single molecule of ATP causes a myosin head to move down the filament in a series of distinct steps, each about 5 nm long. Yanagida cannot rule out

▶ that the myosin head detaches from the filament momentarily during these steps, but he argues that if this happens it is too fast for the myosin to hydrolyse another ATP molecule, and that the net effect is one of loosely coupled movement.

Necks on the block

The problem is that most of the other groups working in the field, using similar imaging approaches to those used by Yanagida, have consistently recorded results that fit the lever-arm theory. They remain convinced that there is a tight coupling between ATP and myosin displacement, and measure this displacement at less than 10 nm (refs 5,7,8). Further evidence for their case comes from structural studies, which indicate that myosin can bend its neck as proposed by the lever-arm model^{9–12}.

At the Osaka symposium, both sides presented fresh results. A key discussion focused on myosin's neck region. According to the lever-arm theory, changing the length of the molecule's neck should alter myosin's power stroke, and hence the displacement induced by a single ATP molecule. But Yanagida predicts that neck length will make little difference.

David Warshaw of the University of Vermont described experiments published last month¹³ in which his group halved the length of the neck of myosin II. Sure enough, this decreased by 40% the average displacement induced by one ATP molecule — and increasing the neck length had the opposite effect. But Yanagida's colleagues described unpublished experiments in which they removed the neck of myosin II altogether, and found no difference in displacement.

Every which way but loose?

Reconciling conflicting results in this field is extremely difficult — especially given the huge investment of time and money needed to create the experimental set-ups. Although everyone in the field acknowledges these constraints, some of Yanagida's competitors feel that his approach makes resolving the debate almost impossible.

"The problem with the loose-coupled thermal ratchet model is that it is difficult to devise experiments to specifically exclude it," says Justin Molloy of the University of York. "The tightly coupled lever-arm idea is simple, predictive and inherently testable because of its more restrictive nature."

Yanagida's glass microneedle experiment, for instance, is controversial. Is the myosin head really positioned on the glass needle as described in the paper? Might there be two myosin heads attached, the fluorescent one, plus one that has lost its fluorescence? If so, this might help explain the multiple steps that Yanagida sees.

But anyone wanting to repeat the experiment faces a formidable challenge. The microneedles were produced by Kazuo Kita-



Field project: Takashi Funatsu's experimental set-up allows muscle proteins to be seen in action.

mura, who says it took him six months of practice before he perfected their manufacture. "No one wants to repeat this experiment in Japan — not even in our own group," says Yoshiharu Ishii, group leader for Yanagida's Single Molecule Processes project.

Yanagida admits that his loose-coupled model can appear a little vague. "But just because I can't explain the whole system doesn't mean my data are wrong," he insists.

In explaining the continuing difference of opinions, Yanagida hints at cultural rifts. In Eastern thought, "it is necessary to pile experimental fact upon fact before asserting anything to be true," he says. This makes Yanagida and his supporters — most of whom are Japanese — comfortable with the absence of a simple, complete model.

But to most muscle biophysicists, this is no excuse for Yanagida's failure to provide a better description of his theory. And they find it difficult to accept his cheerful admission that he rejects the tight-coupling model on intuitive and aesthetic grounds. "It's so boring," he says.

Yanagida invokes his original training as a semiconductor physicist. Transistors, he explains, are fast and simple. "Biological molecules are not that simple," he says, holding his elbow with one hand and pumping it from side to side in imitation of a lever. "They are too soft, too flexible." He also questions the significance of the lever-arm camp's structural studies. "They cannot tell us about function," he argues.

Confusion reigns

Given the difficulty of repeating Yanagida's experiments, many of his competitors complain that they are left to grapple with unsalable data shrouded in a ghost-like theory. One researcher at the Osaka meeting likened him to the US Second World War general George Patton, who gained a reputation for making relentless advances, leaving his col-

leagues to sort out the mess left behind in his wake. "Sometimes I wonder if he's really looking for answers or just out to cause trouble," observes another biophysicist.

Despite the exasperation felt by many in the field, the arguments do not seem to have become tainted with personal animosity. Yanagida is amiable and generally well-liked. And at the Osaka meeting, he maintained a light-hearted playfulness even while disagreeing strongly with some of his peers. Nevertheless, his steely determination becomes clear when asked why he continues to swim against the tide of scientific opinion. "Because I'm right," he responds.

Most biophysicists agree that Yanagida has so far been a force for good, his charisma and technical skills helping draw money into the field. But some researchers fear that the field is becoming mired in an ultimately unproductive debate.

Perhaps the best summing up of the current state of play came in the Osaka symposium's closing speech. "I came here confused about actin and myosin," said Nobel laureate Andrew Huxley of the University of Cambridge. "Now, I am still confused, but at a higher level."

David Cyranoski is *Nature's* Asian-Pacific correspondent.

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Beefing about the risks posed by the French BSE epidemic

Sir—In my capacity as chairman of the UK Food Standards Agency, I read Christl Donnelly's paper (*Nature* **408**, 787–788; 2000) while it was being prepared for publication, and would like to comment on issues of food safety and policy raised by Donnelly's conclusions. Although the study of the French BSE epidemic is made in the context of the situation in the United Kingdom, there are some points that may have broader implications for the European situation as a whole.

Following the 4 December meeting of the Agriculture Council, the European Commission has announced a series of new measures which will have a significant impact in reducing risk in the future. These include a complete ban on the use of mammalian meat and bone-meal for all livestock, the exclusion of all bovine intestine from the food chain and the introduction of an over-30-month (OTM) rule for all member states. Cattle over 30 months will only be allowed into the food chain if they have been tested for BSE and found negative.

This year, the United Kingdom has had ten times as many cases of BSE as France, and yet, according to Donnelly, the current risk of eating an animal close to developing clinical BSE is substantially higher in France than in Britain (52 such animals going into the food chain in France versus 1.2 in the United Kingdom). The explanation for this is that Britain, uniquely, has not allowed any animals over the age of 30 months to go into the food chain, and virtually no animals are close to clinical BSE by this age. The studies of tissue infectivity carried out so far show that most infectivity occurs in animals within the last 12 months before clinical symptoms appear. Almost all infectivity in these animals is removed in the "specified risk material" that is excluded from the food chain.

Donnelly's conclusions depend on three key assumptions: (1) that the age distribution for the onset of clinical BSE in France, reflecting a combination of the incubation period and the age at infection, is the same as in the United Kingdom; (2) that French and UK cattle populations have similar survivorship curves; (3) that 100% of clinical cases were reported in France in 2000, with under-reporting in earlier years, as was the case in the UK. The assumption of complete reporting this year may prove to be optimistic, in which case, risk from French beef will increase roughly in proportion to the degree of under-reporting this year (in other words,

50% reporting would double the risk).

Because of the uncertainties about these three assumptions, especially the third, it is better to think of the relative risks as being an order of magnitude higher in France, rather than to focus on precise numbers. It is also important to keep the risks in perspective. Under Donnelly's assumptions, the risk of consumer exposure to animals close to clinical infection in the food chain in France today is similar to the risk in Britain, say, three or four years ago. The United Kingdom's risk is going down as the epidemic declines.

For consumers of French beef in other European Union countries, the risks will be the same as for French beef in France. However, in order to look at the risks to British consumers from eating imported French beef, one must make an assumption about how effective enforcement of the OTM rule is for imports.

If the OTM rule is fully enforced, Donnelly estimates that the risk from French beef imported to Britain is essentially zero (no cattle within twelve months of developing clinical BSE). If the rule is not fully enforced, the risk scales in proportion to the frequency of breach. Given informal indications to the Food Standards Agency from local authorities that the rule is generally followed in Britain, Donnelly's example of 75% enforcement seems reasonable, and yields a relative risk from French imports of the same order of magnitude as the estimate for Britain's domestic beef.

Donnelly's study (accepting its assumptions) indicates that there is no risk case for a ban on beef imports from France to the United Kingdom—even though this would be a populist move in Britain, given that the French banned UK beef in spite of their own higher risk. It also highlights the importance of effective enforcement of the OTM rule for protecting consumers in Britain, and shows why the European Commission's new plan for an OTM-like rule (the exemption being tested animals) throughout Europe is a key step forward.

Although Donnelly focuses on French carcass meat, comparable if not greater risks to UK consumers could arise from the import of meat products (such as pâtés and salamis) which usually contain beef, as it is difficult to ascertain the age and provenance of any cattle-derived contents.

The precautionary principle, unfortunately, has come to mean all things to all lobbyists. But in the European Commission's view it should start from the available evidence, acknowledging uncertainties; it should be consistent; it should be proportionate to the risk; and it should require frequent reassessment of the risks in the

light of new evidence. Against this background, the UK Food Standards Agency will continue to update its assessment of risk and consequent need for action. However, the European Commission's decision to introduce the new EU-wide measures (including an OTM-like ban) will, when implemented on 1 January 2001, enhance protection against BSE risks for consumers in all member states.

John Krebs

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Acid test finally wiped out vitalism, and yet ...

Sir—Sunetra Gupta in her Millennium Essay "A victim of truth" (*Nature* **407**, 677; 2000) astutely points out the dilemma in which the Swedish chemist Jöns Berzelius found himself when his student Friedrich Wöhler declared in 1828 that he could make urea, a typical product of living organisms, from inorganic sources.

Gupta describes Berzelius's antagonism to the atheistic materialism that abandonment of vitalism would bring.

There was, however, another factor. An important reason that vitalism did not immediately disappear after Wöhler's discovery is given in J. R. Partington's textbook *A History of Chemistry* (Macmillan, London, 1961).

Wöhler synthesized urea from ammonium cyanate. The cyanate was obtained from cyanide, which in those days was made from ferrocyanide which in turn was extracted from the wastes from tanning factories. Thus, to an adherent of vitalism, the urea had not been derived from purely inorganic sources but had a vital component.

The death-knell of vitalism in chemistry was sounded in 1845 when Hermann Kolbe showed that acetic acid, a common end product in living organisms, could be "composed by synthesis from its elements". This was the first use of the word 'synthesis' in a memoir on organic chemistry.

Sidney Toby

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... we need a metaphor to explain life's mystery

Sir—Sunetra Gupta, in her poetic and accurate Millennium Essay on vitalism, concludes that "we remain inclined to believe that the analysis of life does not

detract from its ultimate mystery”.

Vitalism as biological metaphor survived for so long, she says, because it provided a basis for retaining our primary experience of life as a mystery at the same time that we decompose the mystery through scientific analysis.

Holding these two ideas together may be seen as a form of cognitive dissonance, a state of mind producing considerable discomfort and mental disorder.

Allegiance to our current metaphor — ‘life is a machine’ — has led to much progress in medicine and agriculture, for example, but also caused great harm to human beings and the worldwide environment.

Many formal mathematical and other scientific arguments deny the machine model in biology, and it might be a good start to reveal these arguments to our students more than we do now.

We could start, perhaps, with Niels Bohr’s worry that “the minimum freedom we must allow the organism will be just large enough to permit it, so to say, to hide its secrets from us”¹, and end with Diethard Tautz’s treatment of a biological equation equivalent to Heisenberg’s uncertainty relationship in physics suggesting that attempts to predict biological function from genetic information may require “experiment on an evolutionary scale”².

Our inclination to believe in life’s ultimate mystery appears to have a declining vitalistic (or any other) force in the machine world of everyday life. Gupta does a real service in reminding us how important metaphor is in science. Just perhaps, we could find something closer to our primary experience of life than our current machine metaphor as we approach the analysis of living things.

A little bit of scientific philosophy and physical theory like this might, if not vitalize, then at least brighten up those deterministic lectures in Molecular Biology 101.

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Ecology needs theory as well as practice

Sir — In his Millennium Essay (*Nature* **408**, 293; 2000), Jim Smith proposes that ecological theory is generally untestable, and therefore that ecology should concentrate less on theoretical explanation and more on finding applied solutions to humankind’s environmental problems.

Although his concern for solving environmental problems is widely shared by theoretical and empirical ecologists alike, Smith’s call for use of Robert Peters’s ‘predictive ecology’ in place of a more conceptually grounded approach runs the risk of leading ecology into a dead end of blind empiricism.

Smith overstates Karl Popper’s influence over the philosophy of science. Science is a much broader and less formally rigorous enterprise than Popper envisioned. In *The Structure of Scientific Revolutions* (University of Chicago Press, Chicago, 1970) Thomas Kuhn, for example, argued that a large component of cultural subjectivity determines the outcome of the scientific enterprise. His concept of paradigm shifts implicitly assumes that political interactions among scientists are at least as important as empirical verification of hypotheses in deciding the outcome of scientific progress. Science can still uncover much about the natural world, even if it is such a culturally dominated social system. Neither Popper’s views nor a less restrictive philosophy of science justifies the abandonment of conceptual work in ecology.

Progress in ecology has been hampered in the past by assuming that complex ecological systems can be adequately described by relatively simple linearized models, but even those who originated this approach realized its limited usefulness. Significant advances in science are not based solely on the success of a model in predicting some unknown phenomenon.

Smith’s example of relativity is a case in point. The real significance of relativity to physics was not its ability to predict the bending of light by large, massive celestial bodies. Rather, relativity revolutionized physics by revealing an underlying conceptual unity to disparate phenomena. Relativity did not supplant classical physics, it simply limited the spatial and temporal scales to which the rich theoretical framework of classical physics could be applied with acceptable precision.

Ecology will make real scientific progress only by developing a rigorous conceptual framework that can be tested with appropriately sophisticated statistical methodology. Ecological systems are indeed complex. Simplistic semi-empirical relationships based on vague, poorly parameterized, linear statistical models of single systems provide only approximate or temporary solutions to environmental problems. They do not determine which problems are of greatest importance or how limited resources might be optimally allocated.

Only a larger, more comprehensive conceptual framework can provide such

guidance. Explorations of ecological theory are nice work and are essential to the progress of ecology as science.

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Pressure to meet current needs hinders science

Sir — Your report (*Nature* **407**, 276; 2000) about the World Bank giving higher priority to science was a welcome reminder that, unfortunately, national and international ‘development’ organizations have given short shrift to the scientific and technological base that is essential to enable countries to prosper.

In December 1992, former US president Jimmy Carter and I, with a distinguished task force from the Carnegie Commission on Science, Technology and Government, released a report entitled “Partnerships for Global Development: The Clearing Horizon”. We underscored the critical importance of science and technology in economic development along with the imperative for global cooperation.

During the 1990s, however, major donors yielded to pressure to use almost all aid resources for distributing food and medicine today instead of improving agricultural productivity and vaccines for tomorrow. Further, developed countries suffered ‘aid fatigue’ because foreign assistance seemed to be a perpetual handout rather than a catalyst for economic independence. Investment in science and education was also marginalized by grave difficulties in creating financial stability with honest, democratic governance under the rule of law.

US secretary of state Madeleine Albright, seeing a growing need for science and perhaps sensing the new rustlings at the bank, recently named a science adviser: Norman Neureiter, a chemist with an extraordinarily sophisticated understanding of how universities and the private sector interact with the public sector to spark economic growth. This long-needed graft of science onto US diplomacy will demand intensive care for many months.

Along similar lines, don’t expect a quick turnaround from the World Bank’s higher priority on science. The bank will have to rethink its mission and operations to decide how global technological change meshes with its economic strategy.

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Programmed for defence

Waging the immunological campaign against the gamut of wily pathogens.

The War Within Us: Everyman's Guide to Infection and Immunity

by Cedric Mims

Academic: 2000. 228 pp. \$39.95, £24.95

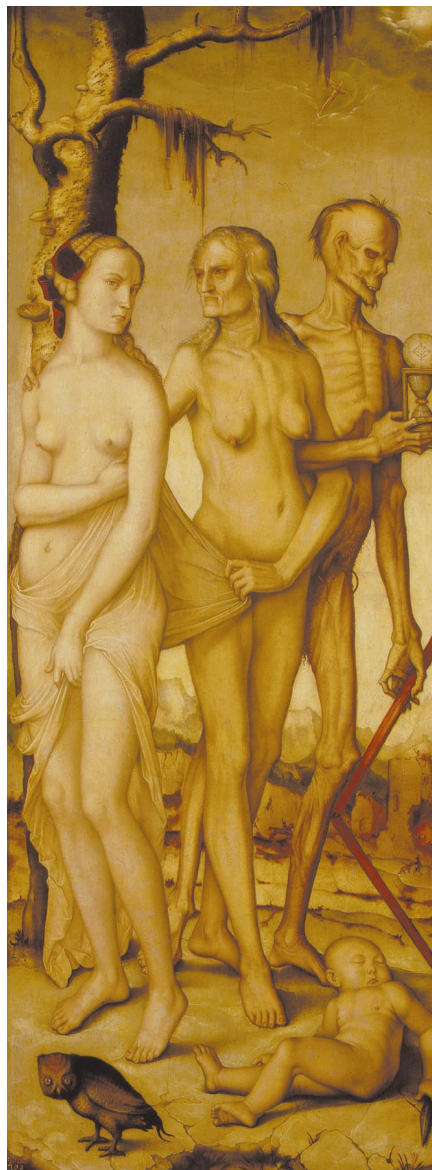
Fred Rosen

On the eve of the presidential election, *The New York Times* found some small space in its editorial columns to praise the Bill & Melinda Gates Foundation for its attempts to lessen the burden of infectious diseases throughout the world. With US\$21 billion in assets, the foundation could easily have distributed computers rather than vaccines to the remotest parts of the developing world. But as Bill Gates said: "The mothers are going to walk right up to that computer and say, 'My children are dying, what can you do?'. They're not going to sit there and, like, browse eBay or something." It was as if Gates had just finished reading Cedric Mims' *The War Within Us*.

Mims has written an attractive and brief overview of the current understanding of immunity and infectious disease. The book is clearly intended for educated lay readers with a minimal background in science. The first third of the book is a reasonably accurate and simple discourse on the components of the immune system and how they work. Although rendered in plain English, it is not oversimplified to the point of introducing gross inaccuracies. The remainder of the text discusses various infectious diseases and the wily pathogens that cause them. Although the way in which immunological specificity is generated must be among the greatest wonders of the natural world, it always evokes striking images of war, and attack and defence, and a whole vocabulary more appropriate to the Pentagon or any other government war ministry. Mims has totally succumbed to this type of imagery. Perhaps he feels it makes the contents more dramatic, and maybe it does.

Scattered throughout the text the reader will find boxed vignettes of fascinating bits of history: how the cook who became known as Typhoid Mary, and who was herself immune to the typhoid bacillus, infected a long succession of her unwitting employers; how Carelton Gajdusek brazenly entered the New Guinea highlands and discovered kuru; how Ignasz Semmelweis, who introduced anti-sepsis into medical practice, died in an insane asylum of the very disease he spent his life trying to eradicate; and how the physician John Snow traced the source of a London cholera outbreak to the Broad Street water pump.

Those of us who grew up before the Second World War have vivid memories of the



Life in all its vulnerability: Hans Baldung's *The Three Ages of Man and Death*.

summer terrors brought on by polio epidemics, of relatives and neighbours dying of tuberculosis, of the ravages of syphilis among the gentry, and of children with whooping cough who were obliged by the health department to wear red armbands. Mims reveals in the course of this book his own medical history, from his mother's death from puerperal sepsis to the time he contracted rift valley fever in the laboratory. His interest in infection and immunity is, it seems, well founded.

Rather disappointingly, no more than two pages of the book are devoted to a discussion of AIDS. Targeted as it is to a lay audience, the book might have been a good

vehicle for saying more about the subject. Nonetheless, the reader is left with a broad understanding of the insidious destructiveness of infectious diseases, and also, no doubt, with a new appreciation of the Gates Foundation's efforts. ■

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Gene games of the future

Engineering the Human Germline: An Exploration of the Science and Ethics of Altering the Genes We Pass to Our Children

edited by Gregory Stock & John Campbell
Oxford University Press: 2000. 169 pp.

£19.95, \$29.95

Veronica van Heyningen

The relegation of sex from being procreational to purely recreational may be premature, but Gregory Stock, one of the editors of this volume, believes that the production of designer babies will eventually take over from normal reproduction.

Altering the human genome in a permanently heritable manner (germline gene therapy) is highly controversial, and in many countries it is prohibited. As with most controversial subjects, though, much can be gained from a thorough discussion of the possible applications, both now and, more speculatively, in the future. This volume is the record of a symposium held at the University of California at Los Angeles in March 1998. It was hosted by Stock, director of the university's Program on Medicine, Technology and Society, and his fellow editor John Campbell, who is professor of neurobiology. The participants, mostly drawn from US academic institutions, fall into three categories: eminent practising scientists; panelists, including James Watson, the co-discoverer of the structure of DNA; and commentators — scientists, ethicists and theologians.

The book paints a futuristic picture of the 'practical aspects' of genome manipulation. Engineering proposals include the use of artificial chromosomes possessing multiple 'docking' sites for introducing several desirable genes simultaneously into recipient cells. Sophisticated chromosomal vectors may be available within a decade. These could, for example, deliver resistance-conferring anti-HIV RNA to helper T cells of

book reviews

the immune system, which are susceptible to attack by HIV. One unanswered question is how the unpaired artificial chromosome will behave at meiosis, when maternal and paternal chromosomes pair up before separation.

Another suggested application of germ-line gene therapy is the prevention of

prostate or breast cancer. According to this stratagem, a toxin gene would be inserted which would be active only in certain tissues and would be expressed only in response to a specific external trigger should the tissue turn cancerous. Similar schemes are envisaged for preventing cell death in

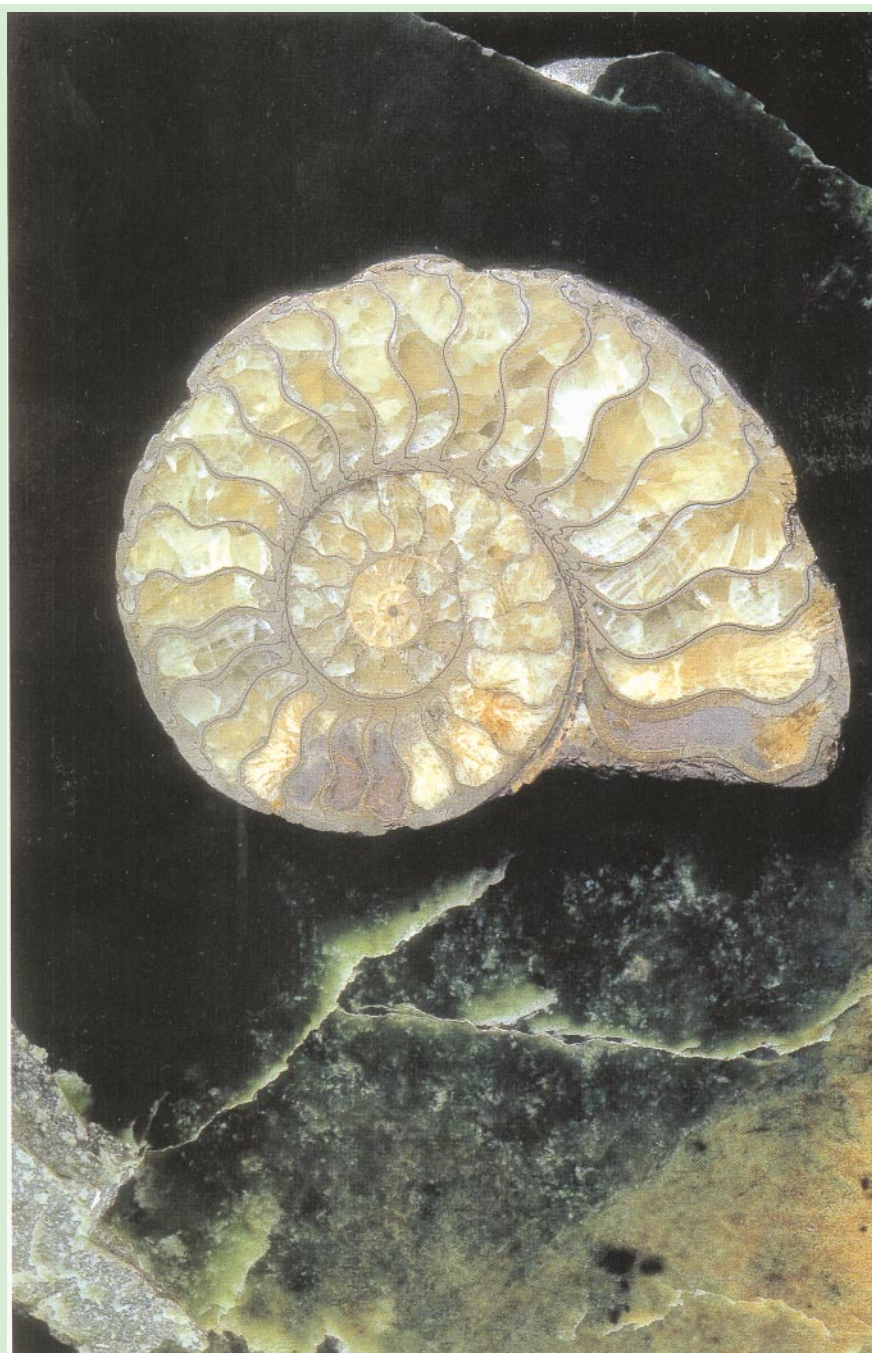
neurodegenerative diseases — once the protective genes have been defined. The resident gene would have to be silenced to ensure the correct 'dosage' and to prevent any interference when the inserted gene was turned on — a difficult molecular control task. But, assuming that such technology can be implemented, there is the problem of how we would select individuals for prophylactic engineering that would be carried out decades before the disease developed.

Lee Hood predicts that gene manipulation will be used to 'enhance' desirable traits such as emotional stability, intelligence and longevity, once we conquer the biology sufficiently to understand how these complex traits are controlled. But this may take 50 years. That gives us time to assess whether we can alter gene regulation with the degree of fine control required in these cases. Much of the technology for manipulating DNA already works in model systems, although there have been very few reports of changes to specific nucleotides — the building blocks of genes — and certainly not simultaneously at multiple gene positions as envisaged for engineering aimed at human enhancement.

Because we are starting to identify genes that affect ageing, it has been suggested that we might be able to prevent ageing by delivering a cocktail of beneficial genes using artificial chromosomes. However, not all participants at the symposium were keen to extend their children's lifespan, since quality of life is perceived to be more important than its duration. And there was some concern about the changes in population dynamics that increased longevity would create.

Jim Watson's view that no novel ethical dilemmas are raised by the concept of gene therapy on the germ line — the set of genes that are passed on to the next generation — is being taken up by some. They believe that the germ line is not particularly sacrosanct; if people think they can safely improve it using new technologies they should be allowed to do so. Watson is particularly eloquent about the moral imperative of seizing any opportunity to redress the genetic inequalities evident in all populations. However, only one or two participants mentioned the tremendous economic costs of such procedures. These would inevitably reinforce and exacerbate the inequalities that already plague us — or, more precisely, that plague the United States, since most of the discussion was centred on the free enterprise, no-federal-interference North American system. The predilection for state regulation in European countries is seen as undue squeamishness which hampers the development of robust, affordable new technology.

Although it seems that the ethical problems raised by germ-line gene therapy can be dealt with in our current moral framework, as a geneticist I feel strongly that the technological difficulties of safe but



Scientifically speaking

Crossing Over: Where Art and Science Meet (Crown, \$27.50) is a collection of essays by Stephen Jay Gould and photographs by Rosamund Wolff Purcell which aims to present science and art in conversation. In this context, a gibbon and Fred Astaire — “brothers under the hair” — are juxtaposed, and a tin toy

illustrates the role of bilateral symmetry in creating complexity. The fossil ammonite shown above, cut, polished and set against a slab of jade, is a symbol of the arbitrariness of extinction and survival — ammonites survived two major mass extinctions, only to succumb to the catastrophe that obliterated the dinosaurs.

effective intervention in the genome have been underestimated. Complications such as genetic control elements that act over a long distance, and overlapping gene domains, make clean genomic intervention difficult. The use of model systems and molecular analysis of human disease is giving us insight into the complexities of gene regulatory mechanisms, and it seems premature to tinker with many of these delicate systems. The amazing thing about mammalian development is not that it sometimes goes wrong, but that it ever succeeds. ■

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Extremophiles in the raw

The Surprising Archaea: Discovering Another Domain of Life

by John L. Howland

Oxford University Press: 2000. 214 pp.
£19.95, \$29.95

P. G. Willmer

Life just keeps on multiplying. When we were very young there were Animals and Plants, which Carolus Linnaeus happily confirmed for us, but which grew into three 'kingdoms' when we had to add on the unicellular things. At school we learned to recognize that unicells came in eukaryotic and prokaryotic versions, those respectively with or without their DNA packaged in a nucleus. A little later, and we had barely learned to love Lynn Margulis' definitive five kingdoms when the molecular lobby started to tell us that the two 'simplest' of these were each in themselves multiples of many kinds of unicellular life-forms. Now the evidence of molecular biologists is forcing us to recognize that not only can life be categorized into five, or even more, kingdoms, but also according to a parallel system of three 'domains' (Eukaryota, Bacteria and Archaea). John Howland's book takes us on a tour of the third domain, the Archaea. Prokaryotic, but not bacterial, they pursue their peculiar lifestyles in the more extreme environments of Earth, as they may have done for perhaps 3.5 billion years.

This biography of their discovery, and the gradual revelation of their unique biochemistry and adaptability, tells a fascinating story. The 'Surprising' of the title hardly does them justice; these creatures are genuinely amazing, and we all need to know more about them. This short book works as an introduction, but it could have been so much better if its target audience had been more carefully defined, without the

jumps from chatty informality (reflected in sub-headings) to stilted academic prose. Some strange choices have been made about what needs careful explaining for the general reader and what is assumed knowledge, while biologists will find the background excessive and the repetition irritating. And instructions on how to obtain archaeal cultures are surely pointless for anyone.

Above all, the book badly needs improved illustration. The few figures are mainly of technical points; photographs don't appear until 80 pages in, and the quality is then so poor as to make them almost useless. The Archaea may not be very photogenic, but surely line drawings would give us a picture image to hang our thoughts around. Descriptions just won't do, unless they are brilliantly written; here, the narrations of the weird archaean cell structures, and their modes of division, are sometimes quite impossible to figure out.

The crucial question for me was how the Archaea differ from the other two domains. The key answer lies with molecular phylogeny; comparative ribosomal RNA analyses put them quite separate from Eubacteria — the other prokaryotes — and the eukaryotes (perhaps nearer the latter). But we are not told about the other key features of Archaea until the fourth chapter, and even then it is easy to get lost; I had to struggle to assemble my own list of what makes these organisms different. This wasn't helped by an unusually high error rate in text and captions; most are trivial and obvious, but when we are told that archaeal lipids contain "either" bonds instead of "ether" bonds, it only adds to the confusion. (And while we are quibbling, only genera and species should be italicized; here, the domains and sometimes the phyla get italics, perpetuating the muddle.)

The book comes into its own, though, in

taking us through the breathtaking range of lifestyles and metabolisms of the Archaea. They are the real extremophiles — living in soda and salt lakes (where they pump chloride and nitrate out across their unique — sometimes purple — version of the cell membrane); in anoxic muds, animal guts and the rocky pillars of deep-sea vents (where they metabolize sulphides and release methane to compound greenhouse warming); and in hot springs in excess of 90 °C (where they manage to keep proteins and membranes functioning against all precedent). One species lives, bizarrely, only in the hot, deep wastes of coal mines, which can have only existed as a habitat for a few hundred years.

Later chapters give us real insight into the importance of the Archaea, and what they may tell us about the Earth's original chimaeric, gene-exchanging life-forms, and possibly about potential life-forms deep within the apparently barren rocks of other extraterrestrial objects.

I had wanted to know more about the Archaea, and now do. And most biologists concerned with life's beginnings, the phylogeny of modern taxa and the adaptability of cellular machineries will appreciate this volume. The final chapter, on the future of the Archaea, should be compulsory reading, not just for those interested in genes, evolution and biodiversity, but also for fuel scientists, chemical engineers and pharmacologists, who may well find that the Archaea have already solved some of their problems for them. But we might have learned more, and more easily, if its text had been shortened to reduce repetition, and the space devoted to tabulations, flow diagrams and drawings. Let's hope that some publisher hears this plea to multiply the media, as well as the taxa. ■

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New in paperback

Air Apparent: How Meteorologists Learned to Map, Predict, and Dramatize Weather

by Mark Monmonier

University of Chicago Press, £11, \$17

"The targeted readership includes both cartographic historians and weather enthusiasts drawn predominantly from the United States. Monmonier's approach is that of the academic cartographer, and this particular slant both gives the book its charm and defines its weaknesses." Huw C. Davies, *Nature* 400, 523–524 (1999)

Classification of Mammals: Above the Species Level

by Malcolm McKenna & Susan K. Bell

Columbia University Press, \$50, £31

"The classification of extant and fossil mammals by McKenna and Bell will be the classic reference

work for all investigators interested in mammalian evolutionary biology." Jean-Louis Hartenberger, *Nature* 392, 350–351 (1998)

The Search for Life on Mars

by Malcolm Walter

Perseus Books, \$15

"Throughout, Walter expresses his feelings about the joy of science, the thrill of the hunt, the difficulty of being sure about anything in the complex geobiological setting, and the frustrations of being half-right, or even wrong, as the work has proceeded." Kenneth Nealson, *Nature* 406, 936–937 (2000)

Speaking in the Air: A History of the Idea of Communication

by John Durham Peters

University of Chicago Press, \$16, £10.50

A cellular cornucopia

Stem cells generate new possibilities for research and therapy.

Margaret Buckingham

Cells with the capacity to re-make parts of the body, or a whole organism, have always excited the imagination. The magic of the process by which a single fertilized egg develops into an embryo, leading to the birth of a new individual, can now be unmasked at the molecular level. The actions of signalling molecules gradually restrict a cell's possibilities, modifying the repertoire of genetic information that it expresses. The universal nature of this selective strategy, with similar molecules acting at different sites within the same embryo in species as different as insects and humans, has been one of the revelations of modern developmental biology.

A major technological breakthrough was the isolation of early embryonic cells that can be replicated *in vitro* while retaining their capacity to contribute to all tissue types. This phenomenon of totipotency opened the way to genetic manipulation of such cells to create mouse mutants and thus test the function of genes during development and in the adult.

Such totipotent cells, together with multipotent cells that can renew themselves and contribute to some, but not all, cell types, were called stem cells long before they had become an experimentalist's tool. Their name evokes the stem of a plant from which leaves and flowers bud. It is also the root of a word that remains after all suffixes or prefixes have been removed. It is here that the linguistic comparison becomes interesting. The stem, from the Latin for a thread, grows up from the root to generate all the other parts of the plant. In German, *Stammzellen* has similar connotations to the English stem cells, but in

French, *les cellules souches* implies something slightly different. The *souche* of a tree is what is left when the tree is cut down — the roots and base of the trunk from which new shoots will grow, eventually leading to new trees. Hence the French word incorporates the idea of regeneration as well as of birth.

Adult animals, even adult vertebrates, can undergo a degree of regeneration. A lizard, for example, can regrow its tail or even an entire limb. Mammals can partially regenerate some tissues, such as liver or muscle, but not others, such as heart. Bone marrow is an adult tissue where replicating precursor cells are present, leading to the repeated generation of different types of blood cell throughout life. Such plasticity is associated

with a susceptibility to malignancy, not seen in tissues such as the heart, where the lock that prevents cells re-entering the cycle is virtually unbreakable.

The existence of adult stem cells capable of contributing to many tissues had been suspected for a long time, but was not backed up by experimental evidence;

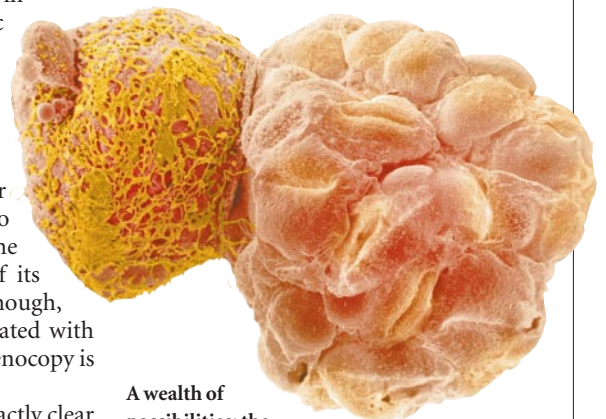
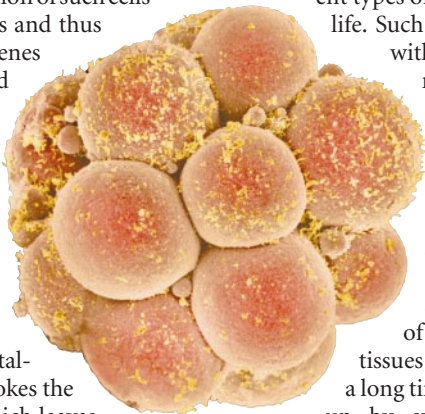
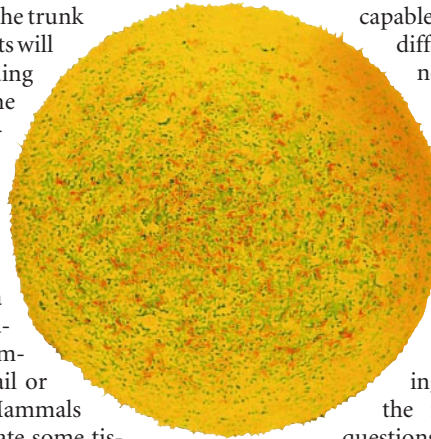
even the regenerative lizard's tail probably depends on the reversion of differentiated cells. Cells are not totally inflexible in the fate that they adopt — a classic experiment in frogs showed that the nucleus of an adult cell introduced into an embryonic cell can contribute to embryo development. The recent production of Dolly the sheep was a further demonstration that it is possible to clone an individual by using the genetic information from one of its cells to make a 'genocopy'; although, despite all the media hype associated with this phantasm of immortality, a genocopy is not necessarily a phenocopy.

In the case of Dolly, it is not exactly clear what the adult cell was. In the past two years, however, thanks to the availability of appropriate molecular markers, it has now been

demonstrated that many adult tissues do contain stem cells. This was first shown in bone marrow, which contains cells capable of colonizing many different tissue types. It is now possible to separate such cells, which have unlimited proliferative capacity and are multipotent, from many different adult tissues. The embryonic origin of these cells and why they remain blind to the instructions that normally restrict cell fate during development are among the fascinating fundamental questions to be answered.

The potential applications of stem cells for the repopulation of damaged tissue is of major biomedical interest. The use of human embryonic stem cells raises ethical issues as well as practical problems, not the least of which is controlling the behaviour of an embryonic cell in an adult environment. For adult stem cells, the issue is why they do not play a bigger role in regeneration normally and how to manipulate them so that they can. At the dawn of the new century, we have found the elusive stem cell. Now we have to master its magic so that new tissue can indeed stem from it, as new shoots grow from the *souche* of the tree. ■

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A wealth of possibilities: the cells in young embryos can differentiate into a multitude of different tissues.

Even adult vertebrates can undergo a degree of regeneration — a lizard, for example, can regrow its tail or a limb.

The holdouts

The contrarians of physics get strung out in Chicago.

George Zebrowski

The annual reunion of string-theory malcontents was held in Chicago on 1 January 2090. Again, the tired theme of the gathering was that real and pseudo-science had somehow reversed roles by the 2050s, with string theory being the ruling dogma of establishment delusionists. The rift among physicists had never been greater or lasted so long. Some science journalists attributed it to the life-extension therapies that had come in during the 2020s.

"Just listen to this," said Chairman Cole-Borner at the opening session, reading from a letter of protest sent to the gathering by Delius Feerce: "String theory, despite experimental support, stands blessedly outside our experiment-bound common sense. Randall-Sundum's so called 'decisive' evidence, the detection of neutrino-warp showers at Cavendish-Lunar 5, was just a pale shadow of the truth of string theory, which rather than needing positive proof is still content to rest on the lack of disproof. Positive-proof seekers tend to find what they are looking for, in the manner of wish-fulfilment. Karl Popper's falsification criterion is more modest — and lack of falsification is what string theory has always had."

"Superstition!" cried Cole-Borner. "They see what they wish and it's consistent with — everything!" He laughed loudly. "Listen, there's more rhapsody."

He read: "The theoretician swims free! Einstein knew how the experiments would turn out, and said he would have felt sorry for the Lord if they had failed. He respected experiment, but speculation, hypothesis and guesses at theory are better beginnings. To experiment is to start with poverty. To start with 'free creations' is to start with riches!"

Cole-Borner looked up from the letter and said, "It's strange that they deny the support of Randall and Sundum's gravitational data, which not only appear to confirm string theory but also a broader cosmology. Their collective delusion is a blot on the history of science. It is not so much string theory that we object to, but the method of its confirmation, which is outright assumption. They reject the embarrassment or triumph that science seeks methodically through experimental experience."

At that moment a holo of Great Hawking's downloaded creative persona intruded into the great space above the hall. "All we ever know is our models," the ghost said from his citadel, "but never the reality that may or may not exist behind the models and casts its shadow upon us who are embedded inside it. We imagine and intuit, then point the finger and wait to see which suspect for truth turns and runs. Our models may get closer and closer, but we will never reach direct perception of reality's thing-in-itself."

"Too much science fiction in the science — that's where it all started," muttered Cole-Borner as the holo winked out.

It was at once replaced by another invader. "Only second-rate scientists make fun of science fiction," Sir Arthur C. Clarke said from his download dais. "Why all this blame? We invent whatever it takes to make a theory work. We even guess the truth. The operationalists don't care why a theory works, so the possible untruth of strings is irrelevant. At the very least strings provide a useful framework to organize our facts and observations, and stimulate further insight into nature's appearance as both continuous and particulate."

"Truth? The phenomena laid bare before us, the user's control panel ripped away to

show the hard and soft wares that run it? That event horizon we embedded creatures cannot cross, much less return to tell the tale ... say, there may be a good story in that!"

He smiled, waved, and winked out.

"Very interesting," Cole-Borner muttered. "But there are still some naive realists among us who wonder why our best theories give the appearance of objectivity gained from outside the system rather than from within it."

What troubled the holdouts most was that string theorists had guessed right and worked backwards to the confirmations — even deriving relativity from string theory in 2025.

It might all have been a matter of style, of how flexibly the ancient insight of granular atomism was to be expressed — from tiny billiard balls and an absolute void, to clouds of particles, to pastas, loose, circular, and cosmic. It was remarkably suspicious how much could be done by mathematical descriptions from 'inside' reality, based on an imaginative, possibly illusory view from 'outside'.

Today's outgoing vacuum-drive starships, repulsive-force drivers, superluminal-ly entangled communications, teleporters, and superbiologies, suggest the approximate validity of String Regime Unification (SRU) and the Infinite Background-Membrane Cosmology (IBMeC) in which it is expressed, and seems unlikely to be only a coincidental match of theory with a transcendent reality. Close enough but incomplete? We can admit that much to the holdouts, in all Gödelian modesty, as we rejoice in the liberation of science from the tyranny of absolute truth.

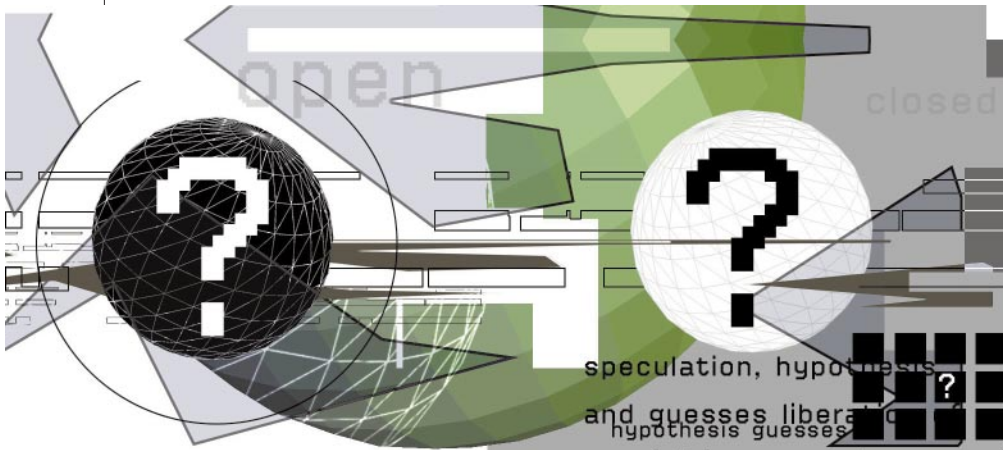
Our sciences exist in a sea of ambiguity. Hermann Bondi (if only he had left us his downloaded persona!) reminds us that the power of science comes from being able to say something, without having to say everything. Absolute truth is too exacting for our creative needs, finite beings that we are.

What a difference a century has made: the force that is accelerating universal expansion now drives our electric power generators! One can only wonder at Hawking's desire to know the mind of God. What was he hoping to do with that knowledge? Break into the codes and rewrite reality itself?

The holdouts say no.

But we say yes.

George Zebrowski received the 1999 John W. Campbell Memorial Award for Best Novel (*Brute Orbits*, HarperCollins). *Cave of Stars* (Harper) is his new novel.



Swimming in Flatsea

Greg Huber

Some of the simplest questions are the hardest to answer. Why flags flap is a long-standing puzzle that becomes easier to solve in a two-dimensional world.

Swimming strings and wind-blown filaments are nowhere to be found in the two-dimensional world created by Edwin Abbott in his sci-fi classic *Flatland*¹. But on page 835 of this issue, Zhang *et al.*² show that at least one visitor to this world, from the even more reduced Lineland, has a few tricks up its slender sleeve. They have managed to observe the periodic flapping of a silk thread suspended in the plane of a fast-flowing soap film. While this experiment might seem odd at first, it actually bears on a host of difficult and unresolved theoretical issues in fluid dynamics — notably the interaction between deformable objects and their surrounding fluid flow. And, like other advances in fluid dynamics, progress in this area is likely to find application in a wide variety of different disciplines. Even the military has shown interest in such matters, in the search for more efficiently propelled micromachines.

Flags flap. This everyday phenomenon contains the essential difficulties of the general problem of elasto-hydrodynamics — the study of deformable bodies in flow. Consider a flag in a mild breeze. The airflow is laminar (streamlined) and non-turbulent. The flag is a flexible object that bends easily and stretches with difficulty — it flutters in the breeze. Why does it not extend straight out in the direction parallel to the streamlines? The confusion over this seemingly simple question is well illustrated by a recent set of ‘answers’ published in a popular science journal³. Unfortunately, all the explanations put forward there are wrong.

Zhang *et al.*² investigate what is essentially a two-dimensional flag waving in a two-dimensional steady background flow. The experimental design is simplicity itself. The authors create a two-dimensional soap channel between two long nylon threads — much as a child might create a sheet of soapy water for blowing bubbles. Soap solution from a reservoir flows down the channel under the force of gravity. This channel set-up was pioneered by Goldburg’s group⁴, and perfected by Rutgers *et al.*⁵. In the current work, one end of a silk thread (playing the part of the one-dimensional flag) is introduced at a fixed location in the flow.

Zhang *et al.* report that their ‘flag’ can develop two stable states. Below a critical length the thread is static and aligned with the flow. For threads longer than the critical

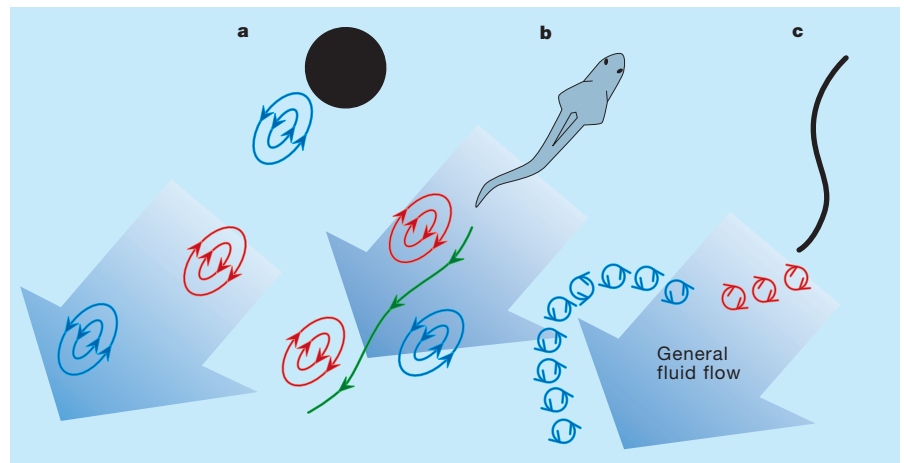


Figure 1 Making flat waves. Vortices created by a disc (a), a fish (b) and a silk thread (c) in a steady two-dimensional flow. Blue indicates anticlockwise-rotating, and red clockwise-rotating, vortices. The green flow line is a propulsive jet in the wake of the fish. The flapping thread studied by Zhang *et al.*² produces a narrow procession of clockwise vortices when it moves to the right, and of anticlockwise vortices when it moves to the left. This is unlike the alternating vortices produced by the disc and fish, or by a straight (non-flapping) thread. Zhang *et al.* also show that their one-dimensional ‘flag’ can be stable and does not always have to flap.

length, a stable flapping state is also possible. In both cases, vortices are produced in the wake of the thread. The vortices trailing the flapping thread are much smaller than the size of each ‘flap’, unlike the vortices created by a disc or a swimming fish, which are comparable to the size of the body (Fig. 1). These results contradict the common belief that a flag is always unstable and will constantly flap in a fluid flow.

On the theoretical side, the party line on the one-dimensional flag problem seems to have been set forth by Lord Rayleigh⁶, who had thought about some elasto-hydrodynamics problems, including the origin of the aeolian tones (the notes produced by wind playing upon a stretched wire). Rayleigh identified simple flags with a surface, or curve in two dimensions, across which the velocity of the wind jumps discontinuously, but where the general flow, left and right, achieves the same uniform velocity some distance away. This is a special case of the so-called Kelvin–Helmholtz instability, in which two distinct fluid streams move relative to each other and the flow at the interface is always unstable⁷.

One might suppose that Rayleigh’s special case is simpler than the general one, but it is not. In mathematical terms, matching the velocities on the left and right is akin to the

problem of tuning the frictional losses on a mass hanging by a spring so that the back-and-forth sloshing of potential and kinetic energies is quickly brought to a stop. In the case of the flag, Rayleigh argued that this special situation, rather than hastening the stability (as in the mass plus spring plus friction system), actually generates an instability, which grows linearly with time. But Rayleigh’s analysis contains some serious flaws, which are highlighted by the experimental evidence reported here.

In the case of the two-dimensional soap film, the silk thread is effectively one-dimensional because the ratio of thread diameter to length is less than 0.0075. This is a fine enough structure. However, a cross-section normal to the plane of the soap film reveals another aspect: the silk thread is a relative leviathan. Its diameter is roughly 50 times the thickness of the soap film. It is not so much lying within the fluid as lording over it, and, as such, its inertia and rigidity are important considerations.

In particular, the results of Zhang *et al.* cannot be explained by Rayleigh’s analysis. Rayleigh’s one-dimensional flag is defined by a velocity jump across a massless boundary, but there is no jump in the pressure of the fluid as would be expected if the boundary were composed of a real material of finite



100 YEARS AGO

Captain Dutton's valuable memoir on the Charleston earthquake of 1886 contains many accounts of the effects of this great earthquake on human beings. Nowhere could they be more vivid than in Charleston itself. "On every side," says one witness, "were hurrying forms of men and women, bare-headed, partially dressed, some almost nude [the earthquake occurred at 9.51 p.m.], and all nearly crazed with fear and excitement... A few steps away, under the gas-lamp, a woman lies prone and motionless on the pavement, with upturned face and outstretched limbs, and the crowd which has now gathered in the street passes her by, none pausing to see whether she is alive or dead...; many voices are speaking at once, but few heed what is said." ... Captain Dutton also gives many records of a feeling of nausea at the time of the earthquake; and, however excitable the observers may have been, these accounts are probably trustworthy, for this is not at all generally known to be a result of earthquake-motion.

From *Nature* 13 December 1900.

50 YEARS AGO

In the account in *Nature* of October 14 of machines which carry out strategic sequences of moves, it is stated: "No machine can learn from its mistakes — to improve the play the programme must be improved". It is, of course, true that a machine cannot learn unless it is provided with a programme or mechanism for learning. But it is quite possible to devise such a mechanism. Machines can be designed to make the best move at each step in a game of noughts-and-crosses or (in theory) draughts or chess. But when playing against a series of human opponents, such a machine may never do much better than draw. A good human player against the same opponents may score more wins by making unsound but more puzzling moves. Can a machine be made to imitate the human player? ... It can, by the inclusion of an empirical or statistical mechanism, in three units. One unit makes the machine experiment with different alternatives each time certain positions are reached; the second unit counts the results and relates them to the alternatives chosen; and the third steers the machine into the lines of play which have been winning most often.

From *Nature* 16 December 1950.

density and rigidity, and under tension. There is also no role for viscosity in Rayleigh's treatment, whereas Zhang *et al.*'s photographs on page 836, and indeed the whole of modern fluid dynamics, indicate the active influence of viscosity at boundaries. This is related to a further problem with Rayleigh's model: that a perturbation of any size leads to instability. That idea is roundly contradicted by Zhang *et al.*'s discovery of a stable static state.

An issue not addressed by Zhang *et al.* is the relationship between the experimental soap-film flow and a theoretical two-dimensional fluid. For a start, air is dragged along with the liquid film, and careful work has shown that this cause of energy dissipation is important for an effective two-dimensional description⁸. And the question of how thickness variations in the soap film are coupled to velocity changes has yet to be settled. Such effects are probably not very important in this experiment because, over short timescales, the thread experiences a nearly laminar flow. But even the simplest examples of vortex generation can hold some surprises^{9,10}.

The graceful undulations of the silk thread are reminiscent of the motion of slender-bodied organisms, such as eels and other types of fish^{11,12}, so it is tempting to suggest a connection. But the filament considered by Zhang *et al.* is not swimming in the conventional sense — if the support arm were removed, the thread would, of course, be carried downstream. The authors suggest that the static state is analogous to a gliding fish, but a comparison of the vortices created in similar flow conditions (Fig. 1)

provides little hope for any immediate biological insights into the mechanics of active swimmers. As von Kármán and Burgers emphasized in their 1935 review article¹³, swimming fish cast off counter-rotating vortices, which induce a jet-like flow running down the middle. The momentum carried off by this jet is in turn related to the forward thrust on the fish.

Zhang *et al.*² have shown that the one-dimensional flapping flag problem is experimentally accessible, and that it yields surprising results. It would seem that a full analysis of real flapping flags requires the development of new aspects of fluid-dynamic theory. Like any good experiment, it should provoke theoreticians, who were thinking about these questions before Edwin Abbott conjured up his two-dimensional world. ■

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Evolutionary developmental biology

Head start

John R. Finnerty

Some neat transgenic experiments show how the evolution of the vertebrate head stemmed, at least in part, from elaboration of controls on pre-existing genetic machinery.

What makes a vertebrate a vertebrate? Arguably, it is the head. All vertebrates have one, but our closest non-vertebrate relatives, the cephalochordates, do not. Clues as to how the vertebrate head came into being can be found among the genes that operate during development. The head's blueprint is somehow written in the genes, and its construction is accomplished by the process of development.

On page 854 of this issue¹, Manzanares and co-workers describe how they have investigated the genes involved, working with gene sequences from the cephalochordate amphioxus (to picture this creature, imagine a small fish that has no bony skeleton, no paired fins and, most importantly, no

head; Fig. 1). Manzanares *et al.* have assessed the head-forming potential of amphioxus gene sequences by introducing them into vertebrate embryos. Surprisingly, it seems that some of these sequences can perform developmental roles that are specific to head development in vertebrates. At first sight this is puzzling, because of course amphioxus has never evolved a head.

The answer to the puzzle lies in the patchwork nature of evolution. Evolutionary innovations blend novelty and antiquity — a relatively small number of serendipitous modifications are superimposed on a larger genetic legacy inherited from ancestors. According to this operating principle, the evolutionary origin of the head cannot

have been based exclusively on new gene sequences, but also on numerous pre-existing sequences. Indeed, the results of Manzanares *et al.* reveal that the invention of the head involved a combination of conservation and elaboration in the gene sequences that regulate development.

Two properties of a gene determine its function in development²: its spatiotemporal expression (where and when the protein encoded by the gene is actually produced), and its interactions with other genes. The spatiotemporal expression of a gene depends in part on *cis*-regulatory sequences. These are sequences of DNA which are not themselves transcribed or copied into messenger RNA, but which regulate the transcription of adjacent genes. Transcription is a necessary precursor to protein synthesis, so regulation of the process is a common mechanism for controlling spatiotemporal gene expression. Modifications to *cis*-regulatory sequences may be the most important proximate cause of morphological innovation during animal evolution³, and such changes do seem to underlie the invention of the vertebrate head.

During development, much of the vertebrate head is derived from two cell populations that do not occur in cephalochordates: neural crest and neurogenic placodes⁴. The neural crest contributes to many of the bones, muscles and nerves of the head and face. The neurogenic placodes contribute to most of the sensory organs, such as the nose and ears, and the associated nerves. Different populations of crest and placode cells contribute to different cranial structures depending on their location along the anterior–posterior axis. For example, the eyes must develop in front of the ears.

But how does a population of cells know its location? Its position along the anterior–posterior axis is specified by the expression of a particular class of developmental genes, the *Hox* genes⁵. Different *Hox* genes are expressed in different places along the anterior–posterior axis, a given *Hox* gene directing the cells in which it is expressed to adopt a certain fate appropriate to its location. Inappropriate expression of *Hox* genes, which can result from mutations in their *cis*-regulatory sequences, can cause an inappropriate structure to develop in a particular location. So the *cis*-regulatory sequences are critical to the spatially appropriate development of cranial structures.

Manzanares *et al.*¹ introduced *cis*-regulatory sequences from amphioxus into the cells of mouse or chick embryos. These transgenic regulatory sequences were fused to a gene encoding a reporter protein, which produced a visible stain in the embryo if the *cis*-regulatory sequences induced transcription. This method allows evaluation of the ability of amphioxus sequences to induce transcription in various vertebrate tissues.

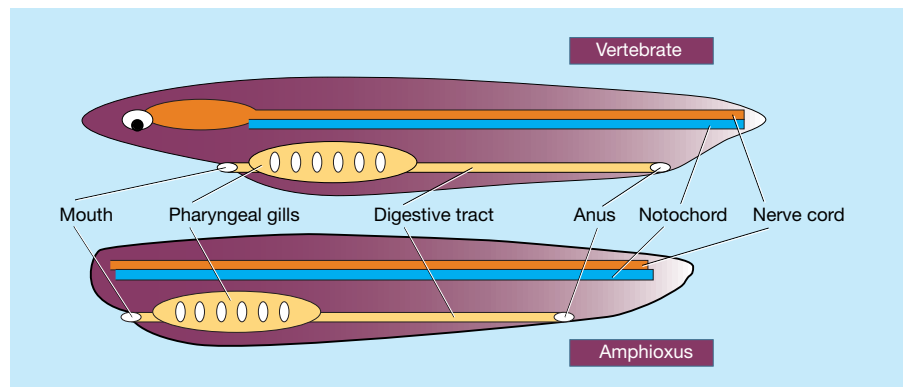


Figure 1 The cephalochordate amphioxus compared to a primitive vertebrate (similar to a modern-day lamprey). The overall construction of the organisms is very similar, with a dorsal nerve cord, a more ventral axial skeleton known as the notochord, and a ventral digestive tract. Both creatures have gills in the pharyngeal region, structures that are designed to capture food or remove oxygen from the water. The notochord extends right to the front of amphioxus, but the vertebrate has a prominent head at the anterior end, extending beyond the notochord. Early in development, human embryos have a notochord that subsequently becomes greatly reduced, giving rise to the discs between the adult vertebrae.

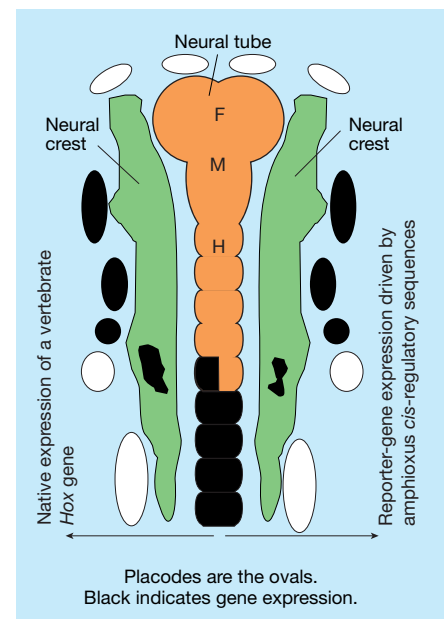
In both mouse and chick cells, the *cis*-regulatory sequences from specific amphioxus *Hox* genes were able to reproduce portions of the native expression of corresponding vertebrate *Hox* genes (Fig. 2). The amphioxus reporter was expressed in the developing hindbrain, the neural crest and the neurogenic placodes, even though amphioxus lacks both crest and placodes. As the authors say, this conserved pattern of expression most likely represents an ancient aspect of *Hox* expression that was present in the ancestral cell type from which neural crest and neurogenic placodes evolved. Where the amphioxus reporter expression differed from the native vertebrate expression, the difference could sometimes be

attributed to specific sequence differences in the *cis*-regulatory region. Unfortunately, it is not yet feasible to study gene expression directly in amphioxus, so the native expression of amphioxus *Hox* genes is not known.

This work¹ illuminates the two forces of greatest interest to the evolutionary biologist: stasis and change. Clearly, much change was involved in the evolution of the vertebrate head. For example, distinct expression patterns, attributable to changes in *cis*-regulatory sequences, are seen in the hindbrain. But there is also evidence of much stasis, including the preservation of ancestral patterns of gene expression, even as new cell types have evolved.

Insights such as these are possible only

Figure 2 Manzanares *et al.*¹ show that gene sequences from amphioxus can perform developmental roles specific to head development in vertebrate embryos. Three tissues are shown in this dorsal view of the head region of a vertebrate embryo: the neural tube, precursor to the nerve cord; the neural crest, which emerges from the dorsal neural tube; and the neurogenic placodes, which are formed from the epidermis overlying the neural tube. On the left is the native *Hox* gene expression driven by the vertebrate's own *cis*-regulatory DNA. On the right is the expression of a reporter gene driven by the *cis*-regulatory sequences of the corresponding amphioxus *Hox* gene. The amphioxus sequences duplicate much of the native vertebrate expression, including that evident in the neural crest and neurogenic placodes. The differences in the neural-tube expression can be attributed directly to differences in the DNA sequence of the *cis*-regulatory regions of amphioxus and vertebrates. The diagram shows the general pattern of cranial *Hox* expression, not of any particular *Hox* gene. Vertebrate *Hox* genes are normally expressed in specific segments of the hindbrain (termed rhombomeres), and in a subset of neural crest and neurogenic placode from the same axial level. F, forebrain; M, midbrain; H, hindbrain.



through the study of evolutionary diversity. An abundance of similar comparative studies will be required to reconstruct the causal connections between genome evolution, developmental evolution and morphological evolution. In the coming years, comparative studies may reveal the genomic and developmental bases for many of the most intriguing evolutionary innovations. For example, comparisons between lampreys and more advanced fishes may elucidate the origins of jaws and complex feeding habits. Comparisons between reptiles and mammals may reveal the origins of the middle ear and sophisticated hearing mechanisms. And comparisons between

reptiles and birds may tell us more about the origins of wings and flight. Clearly, there is no shortage of stimulating questions for the practitioners of evolutionary developmental biology.

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Condensed-matter science

Five-fold symmetry in liquids

Frans Spaepen

A complete description of the structure of simple liquids is missing from our understanding of matter. But new observations show that liquids contain many configurations with five-fold symmetry.

Understanding the structure of simple liquids is a fundamental, unsolved problem in the mathematical and physical sciences. Attempts to describe liquids as disordered crystals have all failed, and their description as dense gases (fluids) remains too complex. The best approach so far, though far from complete, is to describe liquids as dense packings of tetrahedral (four-sided) building blocks. On page 839 of this issue, Reichert *et al.*¹ report the first direct evidence for such polytetrahedral structures in a monatomic liquid trapped at a solid interface.

A complete picture of the short-range order in a liquid (between nearest neighbours) requires knowledge not only of the number and length of the bonds, but also of their directions. Consider, for example, the two 13-atom clusters shown in Fig. 1a and b. Both consist of 12 atoms surrounding a central one at an equal distance, but the two forms are quite distinct. The cuboctahedral configuration (Fig. 1a), in which the bonds form eight tetrahedra and six half-octahedra (composed of four triangles on a square base), makes up the face-centred cubic packing found in simple crystalline structures. The icosahedral configuration (Fig. 1b) consists of 20 tetrahedra, and is an important building block of the polytetrahedral model for monatomic liquids.

The atomic structure of a liquid changes over space and time, so conventional scattering experiments using X-rays or electrons or neutrons provide only directionally averaged information — specifically the distribution of interatomic distances. Such data already favoured the polytetrahedral

model, but Reichert *et al.*¹ provide valuable direct evidence for it. They captured some of the polytetrahedral configurations in liquid lead by aligning them against a crystalline silicon wall. They observed the characteristic five-fold symmetry of the bonds from the scattering of totally internally reflected X-rays, which are sensitive only to the structure of the interface.

Finding a simple structural description of liquids, such as ‘periodicity’ for crystals or ‘sparsity’ for gases, is a persistent challenge in condensed-matter science. It is now accepted that a liquid is not a heavily defective crystal or a random assembly of microcrystals, but a well-defined phase in its own right. This was demonstrated most dramatically in the late 1940s by Turnbull² when he showed that many simple liquids could be supercooled far below their freezing points

without crystallization occurring. This is possible only if the liquid structure is fundamentally different from that of a crystal. That crystals can be substantially superheated later reinforced the idea of a fundamental structural discontinuity between the crystal and liquid states.

Turnbull’s observation led Frank³ to suggest that the structural difference between crystals and liquids arises from liquids having polytetrahedral short-range order. Specifically, he pointed out that, if the interatomic forces act between the centres of the atoms, an icosahedral cluster of 12 atoms surrounding a central one (Fig. 1b) is more stable than a cuboctahedral cluster (Fig. 1a).

The face-centred cubic structure of many crystalline solids (Fig. 1a) maximizes the long-range density of closely packed spheres. Polytetrahedral packing can be viewed as an attempt to maximize the short-range density of a structure. The densest local configuration that can be created with hard spheres is a tetrahedron. Five tetrahedra can be packed around a common edge (shown in red in Fig. 1c), but they leave a gap of about 7°. Twenty tetrahedra can also be packed around a common point, or vertex, to form an icosahedron, which can also be thought of as 12 interpenetrating five-fold rings.

Five-fold symmetry is incompatible with long-range periodicity, so the polytetrahedral short-range order favours disordered or amorphous structures. The model that has been most successful at explaining scattering data from liquids is Bernal’s dense random packing of hard spheres^{4,5} and its computational successors⁶. Analysis of the short-range order in these models reveals ubiquitous polytetrahedral packing, in particular five-fold rings.

Moving the gap between the tetrahedra in a five-fold ring (Fig. 1c) requires very little energy. The thermal disorder of a liquid, the ease by which it flows, and the rapidity by which its atoms diffuse can be qualitatively understood by the redistribution of these gaps. The gaps are there because it is impos-

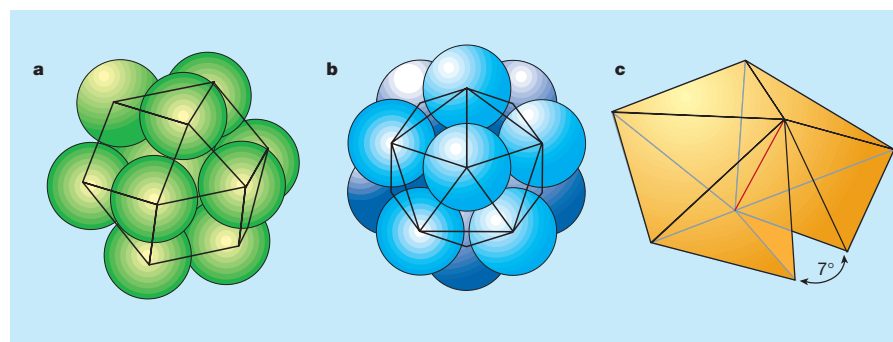


Figure 1 Structural building blocks. Twelve atoms surrounding a central one can form: a, a cuboctahedral arrangement, as in a face-centred cubic crystal; or b, an icosahedral arrangement, as found in a liquid. c, A ring formed by five tetrahedra sharing an edge, leaving a gap with an angle of 7°. The work of Reichert *et al.*¹ suggests that five-fold symmetry is ubiquitous in the structure of liquids.

sible to fill space completely with identical regular tetrahedra. These gaps are akin to those left in two dimensions when trying to tile a surface using regular pentagons. In the latter case, the gaps can be closed up by 'curving' the plane into a sphere, in which case twelve pentagons come together to form a regular dodecahedron. Similarly, regular tetrahedra can be made to pack perfectly in a higher-dimensional space — onto the curved three-dimensional surface of a four-dimensional polytope. Mapping this structure into ordinary three-dimensional space requires the systematic introduction of defects (related to the gaps)⁶, and the diffraction pattern calculated from such a model is in good agreement with experiment^{7,8}.

The experiment by Reichert *et al.*¹ suggests that studying polytetrahedral structures and their defects is a promising route towards understanding the structure of liquids. But much remains to be done. The polytetrahedral polytope used in theoretical studies contains only 120 atoms — only a small part of the bulk liquid. We would also like to be able to use the defects to enumerate all possible configurations of the liquid, and hence to calculate its entropy and other thermodynamic properties. A detailed picture is needed of how the defects allow the local shear that causes viscous

flow, or how they affect atomic diffusion.

And last but not least, the solid–liquid interface, of the type studied here, remains poorly understood. The interface between a crystal and its own melt controls both the nucleation and the growth of crystals — important for all industrial solidification processes, as well as for glass formation. Lack of knowledge about this interface is the largest bottleneck in quantitative modelling of solidification processes⁹. The polytetrahedral approach has been used to create structural models for the crystal–melt interface, and to estimate its thermodynamic properties. The X-ray technique developed by Reichert *et al.*¹ is a useful tool for the direct structural investigation of a variety of crystal–melt interfaces. ■

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that RNA can catalyse transesterification reactions by a mechanism in which metal ions that interact with specific atoms in the RNA activate the attacking nucleophile and stabilize the leaving group (reviewed in ref. 5). But in the context of the spliceosome, it is extremely difficult to distinguish between the relative contributions of RNA co-factors and proteins, both of which are essential (see, for example, ref. 6).

Unlike the cellular protein-translating machinery — the ribosome — the spliceosome is assembled anew on each substrate. Five snRNAs — U1, U2, U4/U6 and U5 — are required for intron recognition and spliceosome assembly. Following initial assembly, a complex series of conformational changes ensues such that the U1 and U4 snRNAs are released before catalysis occurs. So, only U2, U5 and U6 snRNAs could potentially participate in the chemical reactions of splicing.

Of these, U6 has consistently emerged as the best candidate to have a direct role in catalysis. Analyses in a variety of systems have revealed that U6 snRNA must act at or near the active site of the spliceosome. Furthermore, U6 is exceptionally sensitive to mutation and modification. Experiments done several years ago showed that replacing a single oxygen atom with a sulphur atom at a specific phosphodiester linkage within the U6 backbone completely blocked splicing (reviewed in ref. 1). Although it is remarkable that such a subtle alteration could have such a dramatic consequence in the context of a huge, multicomponent complex, the mechanistic basis for this effect could not be established.

Yean *et al.*³ have now looked further at the role of the phosphodiester backbone of U6 and, importantly, their results are amenable to mechanistic interpretation. Each phosphodiester linkage contains two distinguishable 'non-bridging' oxygen molecules, designated R_p and S_p (Fig. 1b). Previous work had established that U6 is inactivated by replacing the R_p oxygen at a specific phosphodiester bond with sulphur. Yean *et al.* show that substituting the S_p oxygen with sulphur at the same position in U6 is equally inhibitory. One well-documented consequence of such substitutions is that they alter the ability of phosphate groups to interact specifically with magnesium ions. So the failure of the U6 snRNA to function might have resulted from its inability to bind a critical metal ion.

To test this possibility, Yean *et al.* determined whether sulphur-substituted U6 could function in the presence of manganese. This metal can, in many cases, fulfil the function of magnesium — but, unlike magnesium, it can interact with sulphur. Remarkably, the function of U6 in which the S_p oxygen was replaced by sulphur was restored when reaction mixtures were

RNA splicing

The case for an RNA enzyme

Timothy W. Nilsen

New evidence suggests that RNA splicing, the removal of non-coding parts of a messenger RNA, is catalysed by an RNA component of the splicing machinery. This component binds a crucial metal ion.

Most messenger RNAs encoded by nuclear genes are synthesized as precursor RNA molecules, in which coding sequences (exons) are interrupted by intervening sequences (introns). For the mRNA to become functional — capable of being translated into protein — the introns must be excised and the exons precisely joined together. The 'splicing' reaction is achieved by the spliceosome, a massive complex consisting of five RNAs and more than 50 proteins. Parallels between the splicing of precursor mRNAs in the nucleus and the self-splicing of introns in some other organelles have fuelled speculation that catalysis within the spliceosome is performed by its RNA constituents (reviewed in refs 1, 2). Proof of this hypothesis would have fundamental implications for our understanding of the evolutionary origin of introns and the machinery that removes them.

On page 881 of this issue³, Yean and colleagues show that one spliceosomal RNA

component — the U6 small nuclear (sn) RNA — specifically binds a divalent metal ion that is required for catalysis of the first step of splicing. It was already known that the spliceosome is a metal-dependent enzyme⁴, so the new results substantially strengthen the case for RNA-based catalysis in splicing.

Splicing is accomplished in two steps, each of which involves a transesterification reaction — the replacement of one phosphodiester linkage with another (Fig. 1a). In RNA, phosphodiester linkages connect one nucleotide to another. Catalysis of transesterification reactions requires both activation of the attacking 'nucleophile' and stabilization of the 'leaving group'. In the first step of splicing, the 2' hydroxyl group of the so-called branch-point adenosine nucleotide (within the intron) is the nucleophile, and the 3' oxygen of the phosphodiester linkage at the 5' splice site (where the 5' exon joins the intron) is the leaving group. Studies of self-splicing introns have shown

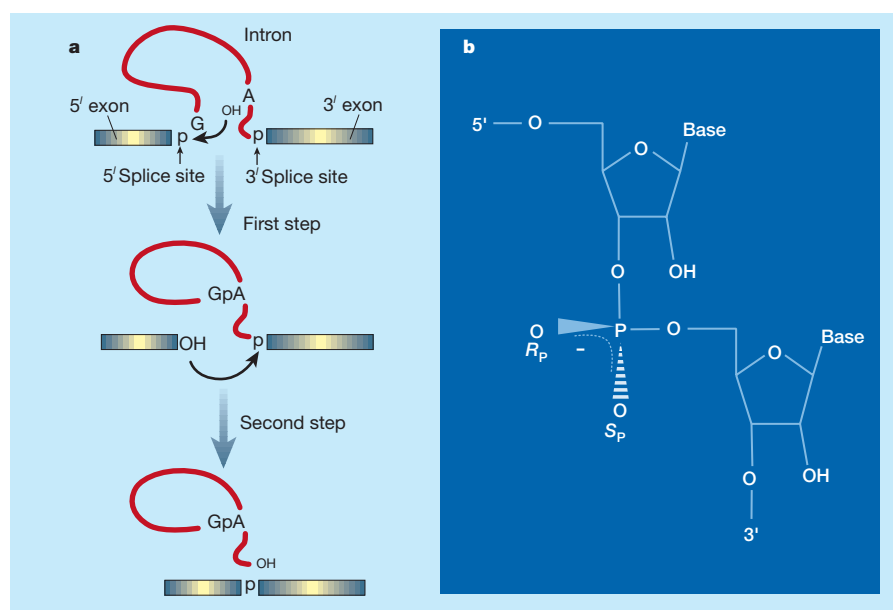


Figure 1 Is the spliceosome an RNA-based enzyme? **a**, The two chemical steps in the splicing of nuclear precursor messenger RNAs. First, the 2' hydroxyl group (OH) of an intronic adenosine nucleotide (the branch-point adenosine, A) attacks the phosphodiester bond (denoted by 'p') at the 5' splice site. Breakage of this bond is accompanied by the formation of a phosphodiester linkage between the branch-point adenosine and the 5' nucleotide (guanosine, G) of the intron. In the second step, the 3' hydroxyl group of the free 5' exon attacks the 3' splice site, yielding joined exons and a free intron. These reactions are catalysed by the spliceosome, an enzyme containing five small nuclear (sn) RNAs and more than 50 proteins. **b**, The details of a phosphodiester bond between two bases in an RNA chain. Each phosphodiester linkage contains two 'non-bridging' oxygens (R_p and S_p) and two bridging oxygens. Yean *et al.*³ show that a magnesium ion bound by the S_p oxygen at one of the phosphodiester linkages in the U6 snRNA is important in splicing.

supplemented with manganese. This provides compelling evidence that the defect observed when sulphur was incorporated was due to a disruption of magnesium-ion coordination. Analogous 'metal-specificity-switch' approaches have previously been used to identify catalytic metal ions used by self-splicing introns, and to show that the spliceosome is a metal-dependent enzyme^{4,7}.

The simplest interpretation of these results is that the metal ion coordinated by U6 participates directly in the chemistry of splicing, either by activating the 2' hydroxyl group of the branch-point adenosine or by stabilizing the leaving group. But this is not the only possible interpretation. Splicing involves many precatalytic steps, any one of which could, in principle, be compromised by the substitution in U6 snRNA. So Yean *et al.*³ devised a clever scheme to accumulate fully assembled spliceosomes, stalled just before catalysis, and show convincingly that sulphur-substituted U6 supports all known precatalytic steps.

These experiments indicate that the metal ion coordinated by U6 may be a critical element of the active site of the spliceosome. When viewed against the extensive backdrop of other circumstantial evidence, the case for RNA-mediated catalysis in the splicing of precursor mRNAs becomes compelling. But is it definitive? Unfortunately not. Metal ions need not be catalytic to be essential, and

metal-ion rescue, even in the simplest of cases, can be difficult to interpret unambiguously (see, for example, ref. 8).

Superconductivity

Geometry spawns vortices

Alan T. Dorsey

The properties of superconductors can be affected by their shape. This effect is increasingly noticeable as the size of the superconductor decreases.

In addition to having zero electrical resistance, superconductors have the ability to expel magnetic fields. It is this fundamental property that allows a permanent magnet to be levitated above a superconducting sample. But under certain conditions the situation changes and magnetic field lines (flux) can partially penetrate some superconductors in a most remarkable way. The result is the creation of magnetic vortices, each carrying one unit (quantum) of magnetic flux.

On page 833 of this issue, Chibotaru *et al.*¹ show that in small superconducting samples, the magnetic flux patterns are greatly affected by the sample's geometry. For instance, when they tried to place three vortices in a square superconducting sample — rather like pushing a triangular peg into a square

Proof that an RNA catalyses splicing would require either the demonstration of splicing in a reaction that does not include the protein components, or high-resolution structural information analogous to that obtained for the ribosome⁹. Although neither result seems imminent, the work of Yean *et al.*³ could conceivably pave the way for such studies. The network of RNA–RNA interactions in spliceosomes assembled with sulphur-substituted U6 might be sufficiently stable to withstand the removal of proteins while retaining manganese-triggered catalytic activity. Alternatively, stalled spliceosomes poised for catalysis might be ideal for structural studies.

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hole — the superconductor spontaneously generated a vortex–antivortex pair, so that the four vortices formed a square with an antivortex in the centre. This surprising arrangement has not been seen in previous studies of disc-shaped superconductors.

Whether or not a superconductor expels an applied magnetic field, H , depends on the field's strength. An ordinary type-I superconductor completely expels the magnetic flux if the applied field is less than a 'critical field', H_c . Above H_c , the sample's superconductivity is destroyed — in other words, the magnetic flux fully penetrates the sample. But in type-II superconductors (which include the copper oxide, or high-temperature, superconductors) the behaviour is more complex because there are two critical fields, H_{c1} and H_{c2} . The

Daedalus

David Jones

David Jones, author of the Daedalus column, is indisposed.

magnetic flux is completely expelled from the sample when the applied field is less than the lower critical field H_{c1} and partially expelled when the applied field is larger than H_{c1} . As before, the sample stops being a superconductor when the applied field is greater than H_{c2} . Quantized magnetic vortices appear in the intermediate region when the applied field is between H_{c1} and H_{c2} .

The vortices can be thought of as long filaments of concentrated magnetic flux, surrounded by a flux-free region of circulating 'supercurrents'. Because the supercurrents do not dissipate energy, the quantized vortices are stable (in contrast to vortices formed by vigorously stirring a bucket of water, which eventually dissipate because of viscous effects). If there are no significant thermal fluctuations or sample imperfections, the vortices arrange themselves into a stable pattern. Because the vortices repel each other, the minimum energy configuration for the vortices is one in which they are as far away from each other as possible. The favoured configuration is a triangular lattice. The characteristics of high-temperature superconductors, such as the maximum superconducting current they can support, are limited by the behaviour of this vortex lattice. So a lot of effort has gone into understanding the patterns of vortices, or controlling them by introducing defects into the superconductor.

So far we have only discussed the bulk properties of superconductors. But what happens when the sample is small and the surfaces or edges play a significant role? A flat surface parallel to the applied magnetic field tends to enhance the superconductivity², so that superconductivity persists near the sample's surface for fields above H_{c2} , and is finally destroyed at a field $H_{c3} = 1.69H_{c2}$. More com-

plex behaviour might be expected to occur for samples with dimensions that compare to the vortex spacing (a micrometre or less).

Advances in nanotechnology over the past ten years mean that it is possible to make such mesoscopic devices and measure their properties. For example, Geim and collaborators³⁻⁵ have uncovered a great deal of exotic behaviour in micrometre-sized discs; and Bolle *et al.*⁶ have used micromechanical oscillators to detect the motion of single vortices in mesoscopic samples. This experimental work has spawned a great number of theoretical studies of vortex nucleation in small superconducting discs and rings^{7,8}. There has also been great interest in using these mesoscopic superconductors as logic elements in a quantum computer⁹. If such a 'qubit' were operated in a magnetic field its maximum superconducting current would depend on the arrangement of vortices, just as in a macroscopic superconductor, so understanding vortex behaviour would be crucial to the operation of the qubit.

The work of Chibotaru *et al.*¹ is different because they have studied magnetic vortices in square superconducting samples. This geometry produces a much richer set of phenomena than the more simple disc geometry studied previously. The group measured the critical temperature (below which the sample becomes superconducting) as a function of magnetic flux in square (2 μm by 2 μm) aluminium samples. (Bulk aluminium is a type-I superconductor, but a thin sample can behave as a type-II superconductor.) The resulting curve has oscillations characteristic of vortex creation (see Fig. 1b on page 833).

To interpret their results the researchers solved the equations that describe the onset of superconductivity in the square geometry,

and got a surprising result: the vortices respect the sample's symmetry by organizing themselves into a square with a fifth vortex at the centre. The nature of this central vortex changes as the magnetic flux is increased, from a vortex, to a giant vortex (which carries a double, rather than a single, quantum of magnetic flux), to an antivortex (responsible for the expulsion of magnetic fields). The theoretical results agree nicely with the researchers' measurements, and the picture they propose for vortex creation is compelling. But imaging the vortices (perhaps using a scanning tunnelling microscope) would provide a more direct and dramatic confirmation.

The results of Chibotaru *et al.* highlight geometry's influence on the patterns of vortices in superconductors, and raise several questions. For instance, what are the dynamics of the vortex nucleation process¹⁰? How do the vortices enter the sample, and what are the barriers⁸ to nucleation? What about other sample shapes — do five vortices in a triangular sample form a hexagon with an antivortex at the centre, or a triangle with a giant vortex at the centre?

It is also possible that the findings of Chibotaru *et al.* might apply to materials that show 'super-behaviour' such as superfluid helium or Bose-Einstein condensates¹¹. These can also flow without resistance and generate stable vortices when rotated in a container. Earlier this year¹², giant vortices were generated for the first time in superfluid helium-3. Chibotaru *et al.* suggest that superfluid helium, rotated in a triangular or square vessel, might generate antivortices. Similarly, the laser fields used to confine Bose-Einstein condensates could be arranged to encourage the production of antivortices in triangular or square traps.

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Plant biology

Volatile defence

Plants can't run away from trouble and have developed sophisticated chemical defences instead. Maize, for example, releases a cocktail of volatile indole and terpenoid compounds when attacked by the beet armyworm caterpillar (*Spodoptera exigua*, pictured). These compounds attract a parasitic wasp, which deposits its eggs in the caterpillar; the wasp larvae then devour the caterpillar.

Writing in *Proceedings of the National Academy of Sciences USA* (online early edition, 5 December), two groups describe their investigations of how maize

produces the substances. Monika Frey and colleagues have identified a gene, *lgl*, that is involved in the synthesis of indole. And Binzhang Shen and co-workers show that another gene, *stc1*, is required for maize to make a sesquiterpene compound (a terpenoid).

Maize releases the compounds only when under attack, so it seemed likely that the genes are activated only temporarily. Using techniques such as treating maize plants with volicitin, an 'elicitor' substance in the caterpillar's saliva, both groups show that each gene is indeed switched on only in response to damage.



Finally, Shen *et al.* look at maize plants in which the *stc1* gene is mutated, and discover that they do not produce a major volatile compound seen in the normal plants.

Amanda Tromans

Obituary

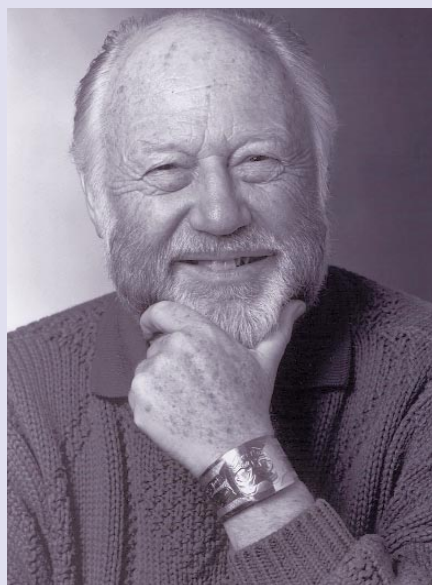
Michael Smith (1932–2000)

Some researchers live their lives by the scientific credo that the best path to creative discovery is an open mind, a stimulating environment and colleagues, and outstanding resources with which to test hypotheses. Such a scientist was Michael Smith, who died on 4 October 2000 at the age of 68. Michael's landmark contribution to science was the development of site-specific mutagenesis, a technique in which small, synthetic oligonucleotides are used to substitute one base pair for another in the DNA of organisms. This approach is now used worldwide, and is crucial in understanding the importance of specific amino acids to the function of particular proteins. The significance of this discovery was recognized by Smith's receipt of numerous prizes and honours, culminating with the Nobel Prize in Chemistry in 1993.

Michael grew up in a working-class neighbourhood in Blackpool, England, and was the only student in his high-school class who went on to study at university. After graduate studies at the University of Manchester, he joined a young scientist, Gobind Khorana, in Vancouver as a postdoctoral fellow, working on nucleotide chemistry. Later, he moved with Khorana to Wisconsin. In 1961, his love of the outdoors led him to return to Vancouver to accept a position with the Fisheries Research Board of Canada, where he combined research into marine biology and into the chemistry of nucleic acids.

Smith's postdoctoral fellowship, together with his sabbatical in 1975 in Fred Sanger's laboratory, provided him with the background and stimulus that were to lead him to the recognition that it is possible to change a single base in a gene, whatever its size. Interestingly, his first paper describing the technique of site-specific mutagenesis was declined by a premier scientific journal. The discovery was clearly ahead of its time. Michael often told this story, particularly to encourage those whose initial report of a scientific advance was not met with unbridled enthusiasm.

Michael was passionate about science, but also dedicated much of his time to the community and country he lived in. Not only did he give away the earnings from his Nobel Prize to causes about which he cared deeply — research into schizophrenia and the development of



Scientist who developed a landmark technique for gene analysis

fellowships for women in science — but he also persuaded provincial and federal governments in Canada to match these awards. He saw no barrier to raising public and government awareness of the importance of basic research to the quality of life of Canadians, and was constantly active in influencing the highest levels of government towards that agenda. After receiving the Nobel Prize, he gave his time, expertise and fame to humanitarian causes — a clear example of the power of individuals in science.

Canada has seen a marked improvement in its research environment and in its support of the early careers of scientists, in large part thanks to Michael's commitment to this goal. He recognized how important it is to ensure that children and students are, at an early age, exposed to the wonder and beauty of science. His story about the rejection of his landmark paper, as well as the fact that he was never a stellar high-school student, gave those he spoke to the belief that anything was possible, as long as you were willing to take risks and pursue your goals diligently.

In the late 1980s, he developed, and persuaded others to support, the idea of a research centre where people with different expertise — in physics, chemistry, botany, zoology and biology — could work together. This led to the

founding of the biotechnology laboratory at the University of British Columbia. He was also the founding director of the Centre of Excellence in Protein Engineering, one of the first eleven Centres of Excellence in Canada.

At the age of 64, Michael was longing to get back to the bench, and took a sabbatical with Maynard Olson at the University of Washington, where he concentrated on genomics and the power of DNA sequencing. With that knowledge, and a firm belief about the future of genomics research and its implications for biology, he accepted an invitation from one of us, Victor Ling, to become the founding director of the Genome Sequence Centre at the British Columbia Cancer Agency.

He was a brilliant recruiter, both there and at the biotechnology laboratory at the University of British Columbia. He often used to explain that the secret was to recruit not the scientist but rather the person. By that, he meant that it was important to understand all aspects of a person — their dreams and aspirations for science, as well as other parts of their lives. That was why he was often to be found at the airport welcoming candidates, and spent a lot of time finding out about their hopes for the future. He was able to converse with his colleagues even about areas totally outside his training or expertise. He would frequently place articles of interest in people's mail boxes, and engage whoever he could talk to in conversations about what excited them in their research.

He was passionately committed to science. But most of his colleagues who spoke at the celebration of his life in Vancouver on 6 November recalled his generosity, friendship, zest for life, and love for his family and three children. He also loved the west coast of British Columbia. An avid hiker, skier, fisher and boater, he was full of tales about the fish he caught, or the pod of killer whales leaping in the air just off his seaside home at Bliss Landing. He will be sorely missed as a colleague, friend and innovator, and as a wise and imaginative mentor in developing and attaining dreams.

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Likely size of the French BSE epidemic

Epidemiological analysis helps in evaluating the potential risks of eating French beef.

The United Kingdom has reported the largest number of cases so far of bovine spongiform encephalopathy (177,490 animals), but native-born cases have been reported in other European countries as well (Republic of Ireland, 524; Portugal, 447; Switzerland, 350; France, 143; Belgium, Denmark, Germany, Liechtenstein, Luxembourg, the Netherlands and Spain, each fewer than 20 cases)¹. Here I analyse BSE-incidence data² from France (Fig. 1a) to estimate the size and course of the epidemic there and find that at least 1,200 French cattle have been infected with the aetiological agent that causes BSE since mid-1987. This suggests that, even under the most optimistic assumptions regarding the incidence of BSE infection in France, an estimated 49 infected animals will have been slaughtered for human consumption in France during 2000.

The risk of BSE entering the food-chain from British beef has been markedly reduced now that cattle slaughtered for consumption are restricted to those under 30 months old (these younger animals are now least likely to have been infected), but more late-stage infected animals are likely to have been slaughtered for meat this year in France, where no age restriction for slaughter has been imposed. (All animals slaughtered after 30 months will be tested from 1 January 2001.)

I obtained a good fit to the age-specific incidence of BSE in France using a birth-cohort model³ (goodness-of-fit *P* value of 0.09, allowing for under-reporting before 2000) with validated parameter estimates obtained from back-calculation analysis of the British BSE epidemic^{4–7}. The results, allowing for under-reporting, indicate that the risk of infection by the aetiological agent for BSE dropped dramatically from 1988 to 1991 (Fig. 1b), perhaps as a result of avoiding meat and bone-meal in cattle feed after the ban was introduced in Britain. (Analysis of the British epidemic has previously shown that the ban was not fully effective when implemented in mid-1988; refs 4–7.)

The subsequent gradual rise in infection risk between 1991 and 1996 may reflect the recycling of infectious material within the French feed industry, as occurred in the BSE epidemics in Portugal and Switzerland^{3,8}. Although the inclusion of ruminant tissue from the central nervous system was banned from ruminant feed in France in mid-1996 (ref. 9), it is too soon to judge the effectiveness of this ban on the incidence of BSE infection.

I estimate that, since mid-1987, 7,300

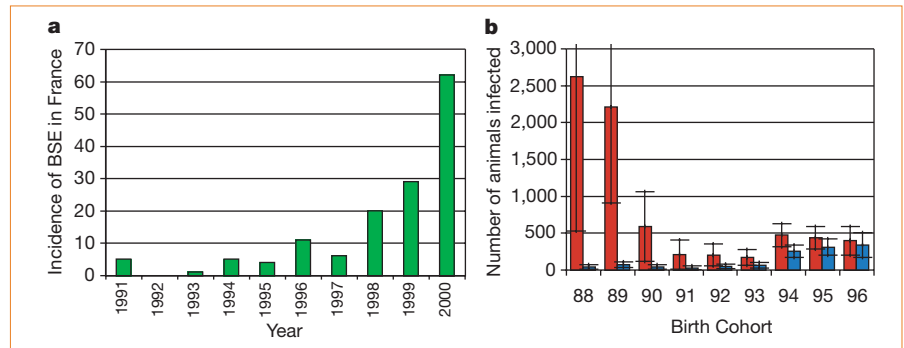


Figure 1 BSE incidence in France. **a**, BSE case incidence by year of clinical onset (confirmed and reported by 17 November 2000; data include only French-born cattle). **b**, Estimated BSE infection incidence by cohort (with 95% confidence intervals) allowing for under-reporting (red) and assuming complete reporting (blue). Annual birth cohorts were defined so that, for example, the 1992 cohort consists of cattle born between 1 July 1991 and 30 June 1992. The age-specific incidence of BSE was modelled by birth cohort, denoted $I(a, c)$ for age a years and birth cohort c , as $I(a, c) = \Delta(a+c) S(a) N(c) f(a)$, where under-reporting is modelled as a time-dependent probability that a BSE case is reported, $\Delta(a+c) = \exp(\alpha + \beta(99 - a - c)) / [1 + \exp(\alpha + \beta(99 - a - c))]$ for $a + c = 99$ and $\Delta(a+c) = 1$ for $a + c = 100$ (ref. 2). $S(a)$ is the probability that an animal survives to age a , $N(c)$ is the number of animals in cohort c infected with the aetiological agent of BSE, and $f(a)$ is the probability (assuming all animals survive to age 18 yr) that an infected animal will experience clinical onset at age a . The parameters α and β were estimated using maximum likelihood assuming that the age-specific incidence of BSE by birth-cohort data arose from a Poisson distribution. The age-at-onset distribution, $f(a)$, (the convolution of the age-at-infection and incubation-period distributions) and the survival distribution, $S(a)$, were taken from validated parameter estimates obtained from back-calculation analysis of the British BSE epidemic^{4–7} and survival distribution of British cattle^{4,7}. In a parallel set of analyses, the observed incidence in the last age category for each cohort ($I(a, c)$ for $a + c = 100$) was boosted by 21% (estimated from the change in $I(a, c)$ for $a + c = 99$ between November 1999 and November 2000) to adjust for confirmation delay, as well as for the onset of clinical cases that have yet to occur because, for example, animals in the 1994 cohort could experience clinical onset at age 6 yr in early 2001. As a result of this correction factor, total infections and the number of infected animals slaughtered in 2000 were roughly 19% greater if allowance was made for under-reporting (and 13% greater assuming complete reporting).

French cattle (95% confidence interval: 4,700–9,800) were infected with BSE. This estimate is reduced to 1,200 (900–1,400) if it is assumed that case reporting is complete; however, under-reporting is highly significant ($P \leq 0.001$) and the steady rise in BSE incidence from 1987 to 1996, estimated on the assumption of complete reporting, is difficult to explain.

Furthermore, independent analysis of cattle imported from the United Kingdom by France and other European countries consistently indicated that the number of reported cases of BSE was fewer than expected¹⁰. Under-reporting was found to be an important feature in the early years of the British^{4,6,7}, Portuguese³ and Swiss⁸ BSE epidemics.

I also estimate that 100 infected animals (81–120) will have been slaughtered for human consumption during 2000 in France, with the slaughter of 52 (42–62) of these estimated to have occurred within 12 months of the clinical onset of BSE. Assuming complete reporting, these estimates are reduced to 49 (37–61) and 24 (16–30) animals, respectively. By comparison, the estimated number of infected animals slaughtered for consumption in 2000 in Great Britain within 12 months of clinical

onset of BSE is 1.2 (0–4) (ref. 11).

Since the announcement in 1996 of the diagnosis of a new variant of Creutzfeldt–Jakob disease (vCJD) linked to BSE, public concern has been heightened regarding food safety generally and the safety of beef in particular. Although an estimated 900,000 British cattle have been infected with BSE^{4,6,7}, the ban introduced in 1996 on the consumption of beef from British cattle over 30 months of age reassured the public regarding potential risks posed by beef consumption.

Current British legislation bans the sale for consumption of beef from animals over 30 months of age from all BSE-affected countries. Thus, if this legislation is fully enforced and the French feed ban has eliminated feed-borne transmission of BSE to cattle born since mid-1996, then there is virtually no risk from French beef consumed in the United Kingdom. Furthermore, even if this legislation were only 75% enforced, then the risk posed by British and French steaks sold in the United Kingdom would be comparable (correcting for the national herd sizes and ignoring regional heterogeneity in incidence). Even in the pessimistic scenario that the incidence of BSE infection in French cattle was not

reduced by the 1996 feed ban but remained constant, I estimate that only two late-stage infected animals under 30 months old will be slaughtered for consumption in France.

In summary, robust cohort-based epidemiological analyses should form a suitable framework for re-examination of the potential risks posed by the consumption of beef from countries with native-born BSE cases.

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Illusions

What you see is what you hear

Vision is believed to dominate our multisensory perception of the world. Here we overturn this established view by showing that auditory information can qualitatively alter the perception of an unambiguous visual stimulus to create a striking visual illusion. Our findings indicate that visual perception can be manipulated by other sensory modalities.

We have discovered a visual illusion that is induced by sound: when a single visual flash is accompanied by multiple auditory beeps, the single flash is incorrectly perceived as multiple flashes. These results were obtained by flashing a uniform white disk (subtending 2 degrees at 5 degrees eccentricity) for a variable number of times (50 milliseconds apart) on a black background. Flashes were accompanied by a variable number of beeps, each spaced 57 milliseconds apart. Observers were asked to judge how many visual flashes were presented on each trial. The trials were randomized and each stimulus combination was run five times on eight naive observers.

Surprisingly, observers consistently and incorrectly reported seeing multiple flashes whenever a single flash was accompanied by more than one beep (Fig. 1a). Control conditions and catch trials (Fig. 1 legend) indicate that the illusory flashing phenomenon is a perceptual illusion and is not due to the difficulty of the task, cognitive bias or other factors.

Figure 1b shows that observers' performance was the same, irrespective of whether a single flash was accompanied by two beeps, or two flashes by one or no beeps, suggesting that the illusory double flash is perceptually equivalent to the physical double flash. This was confirmed by the testimonies of the observers after the exper-

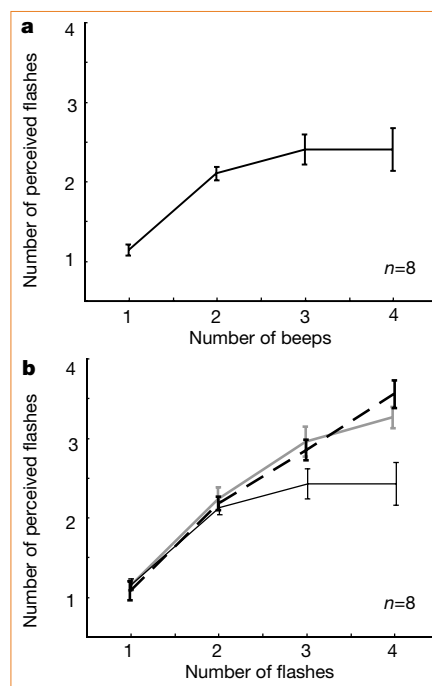


Figure 1 Illusory flashing. **a**, Perceived number of visual flashes by eight observers plotted as a function of the number of auditory beeps for a single flash. The number of perceived flashes did not increase linearly with the third and fourth beeps because they fell outside the optimal window of audiovisual integration, as revealed by our next experiment. **b**, Perceived number of flashes by eight observers plotted as a function of the actual number of flashes presented for trials with no sound (dashed line), and trials with single beeps corresponding to catch trials (grey line). Observers performed the task very well in the absence of sound (dashed line). The results of the catch trials (grey line) confirm that the observers' responses were not determined by their auditory percepts. The curve in **a** (for a single flash) is superimposed for comparison. Further details can be obtained from L.S.

iment. The performance of non-naive subjects (results not shown) indicated that the illusion persisted even when subjects were aware of the fact that the disk was actually flashed only once.

We next investigated the temporal properties of this illusion by varying the relative

timing of visual and auditory stimuli. The illusory flashing effect declined from 70 milliseconds separation onwards. However, illusory flashing occurred as long as the beep and flash were within approximately 100 milliseconds of each other, which is consistent with the integration time of polysensory neurons in the brain^{1,2}.

Our results indicate that the illusory flashing is caused by an alteration of visual perception by auditory stimuli. The modification of the visual percept by sound, however, was not categorical. It was rather selective, as sound did not have a fusing effect when multiple flashes were accompanied by a single beep. We suggest therefore that the direction of cross-modal interactions is partly dependent on the type of stimulus. Consistent with previous observations in other modalities³, we propose that the percept of a continuous stimulus in one modality is rendered more malleable by a discontinuous stimulus in another modality than vice versa.

The influence of auditory cues on visual perception has been demonstrated in other settings, in which perceived visual intensity is affected by the presence of an auditory stimulus⁴. This influence, however, is quantitative and does not alter the phenomenological quality of the percept. Others have shown that the perceived direction of ambiguous visual motion is influenced by auditory stimulation⁵.

Our results extend these previous findings by showing that visual perception can be qualitatively altered by sound even when the visual stimulus is not ambiguous. The conditions under which this alteration occurs — the stimulus configuration and the task — are very simple. The illusion is also surprisingly robust to variation in the many parameters we manipulated (including disk eccentricity and contrast, spatial disparity between sound and flash, shape and texture of the flashing pattern, flash and beep durations). The simplicity and robustness of the illusory flashing phenomenon indicate that it reflects a fundamental and widespread property of polysensory mechanisms in the brain.

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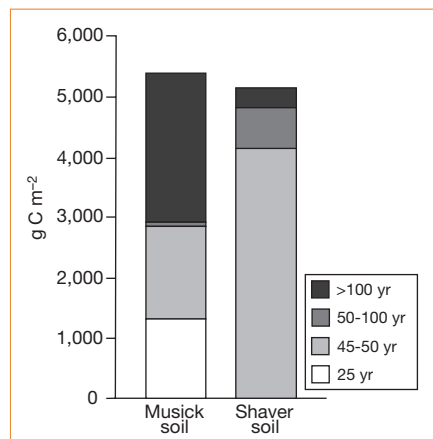
Biogeochemistry

Soil warming and organic carbon content

Soils store two or three times more carbon than exists in the atmosphere as CO₂, and it is thought that the temperature sensitivity of decomposing organic matter in soil partly determines how much carbon will be transferred to the atmosphere as a result of global warming¹. Giardina and Ryan² have questioned whether turnover times of soil carbon depend on temperature, however, on the basis of experiments involving isotope analysis and laboratory incubation of soils. We believe that their conclusions are undermined by methodological factors and also by their turnover times being estimated on the assumption that soil carbon exists as a single homogeneous pool, which can mask the dynamics of a smaller, temperature-dependent soil-carbon fraction. The real issue about release of carbon from soils to the atmosphere, however, is how temperature, soil water content and other factors interact to influence decomposition of soil organic matter. And, contrary to one interpretation³ of Giardina and Ryan's results, we believe that positive feedback to global warming is still a concern.

Climate has long been believed to affect the accumulation of detrital carbon and nitrogen⁴ — cool conditions and poor aeration in wet soils can impede decomposition⁴, and soil carbon stocks are highest in cool and moist biomes and lowest in hot and dry biomes⁵; climate also affects plant productivity and the input of carbon to the soil. To determine decomposition (carbon output), a simplistic one-pool model (soil carbon stock/(field-measured soil respiration minus estimated root respiration)) can be used to calculate average turnover times (soil carbon stock/decomposition rate) of total soil carbon⁶. Although this one-pool model is not without problems (see later), a trend of increasing average turnover times with declining mean annual temperature and increasing latitude is apparent in these field data.

Temperature should not be viewed in isolation. For example, temperature affects evapotranspiration, which affects soil water content, which influences decomposition of soil organic matter (SOM)^{1,6}. Climate is only one of a hierarchy of factors controlling decomposition, including clay mineralogy, chemical properties of leaf and root litter, activity of macrofauna and micro-organisms, and disturbance⁷. Giardina and Ryan's proposal² that temperature has no effect on the turnover of soil carbon is based on their search for only a single factor in this complex matrix and also on, in our view, inappropriate tools and sampling



design (involving a type II statistical error).

Giardina and Ryan used changes in ¹³C content of SOM following conversion of C₃ vegetation to C₄ vegetation, which necessitates different degrees of site disturbance. Ploughing causes rapid, large changes in decomposition, exposing SOM previously protected inside soil aggregates^{8,9}. Conversion of forest to cattle pasture, however, causes only moderate soil disturbance and small changes in SOM decomposition¹⁰. The degree of disturbance may thus have a larger effect on calculated turnover times than either temperature or the time elapsed since disturbance.

Giardina and Ryan base their estimates of soil-carbon turnover times on the assumption that SOM can be represented as a single homogeneous pool, an approach that has yielded mixed results^{2,6} because it ignores the widely varying age (from months to millennia)¹¹ of carbon in SOM. The proportion of SOM cycling over millennia ranges from zero to over 50%, depending on mineral type, clay content and soil depth. This variation has a large effect on the calculated turnover times when soil-carbon fractions that cycle at different rates are averaged together¹¹.

Figure 1 illustrates this point, showing results from radiocarbon analysis of two soils sampled along an elevation transect in the Sierra Nevada mountains of California¹², where turnover times of fractionated carbon pools in each soil range from 25 to over 1,000 years. Although the total soil carbon is comparable in both soils, the soil at lower elevation (Musick soil), which experiences higher annual temperatures and slightly less precipitation (not higher²), contains less labile carbon with a faster turnover time than the higher-elevation (Shaver) soil. Decomposition of the SOM fractions during a one-year incubation would sum to similar amounts of CO₂ for the two soils, hence the single-pool model of Giardina and Ryan would ascribe similar turnover times to both soils, obscuring the faster cycling of the smaller labile fraction of carbon in the warmer, lower-elevation soil.

Figure 1 Radiocarbon estimates of turnover times of carbon fractions of two soils on an elevational gradient with similar parent material, vegetation and disturbance history¹². Fractions were separated by density and hydrolysis for each soil depth¹². The CO₂ that would be evolved during one-year incubations (98 and 92 g C m⁻² yr⁻¹ for Musick and Shaver soils, respectively) was calculated from carbon stocks and turnover times. Dividing respired CO₂ by total soil carbon, as Giardina and Ryan² do in their one-pool model, yields nearly identical turnover times estimates for the two soils (53 and 54 yr for Musick and Shaver soils, respectively). However, the cooler Shaver soil contains twice as much carbon with turnover times of about 50 yr and the warmer Musick soil has a small but important pool that cycles more rapidly.

Laboratory incubations, as used by Giardina and Ryan², involve procedures such as sieving and mixing, so are not comparable to decomposition *in situ*. In some soils, the small amount of easily decomposable humus can be spent before the end of a one-year incubation, so differences in calculated turnover times among samples may reflect different substrate limitations rather than temperature sensitivity¹³; they may also reflect differences in sampling depths, which affect the proportions of labile and non-labile carbon.

Rather than averaging turnover times of total extant SOM, it is the carbon that has already left the soil that may reveal the latitudinal importance of temperature on SOM dynamics — for example, slash-and-burn agriculture in the tropics must be abandoned after a few years as the small amount of labile soil humus releases nutrients for only a short time. By contrast, agricultural productivity in a northern temperate climate was maintained for decades without extra nutrients after ploughing soils with a similar sand content, but with more humus that decomposed gradually in the cooler climate⁹. Variations in mineralogy and aggregate stability also contribute to these differences.

The temperature sensitivity of soil carbon subject to climate change has been tested by fractionating SOM into pools that can be compared along climate gradients^{9,11,12}. *In situ* experimental soil warming also increases soil CO₂ emissions unless it provokes drought¹. Increased emission was limited to a few years after warming treatments began, indicating that only a fraction of the soil carbon is temperature-sensitive and supporting our assertion that carbon dynamics should be studied using multi-pool models.

In a field study along climate gradients¹⁴, plant biomass increased in all the EUROFLUX sites, even those with a carbon budget close to neutral and one losing carbon annually — the site losing carbon was drained, exposing anaerobic soil layers to decomposition. Soil carbon loss also happens where warming helps to dry wetlands

or thaw frozen soil¹⁵. The EUROFLUX sites with almost neutral carbon balance are at high latitudes where significant warming has occurred, which may provoke loss of soil carbon at a rate comparable to plant biomass gains¹⁵.

Unlike Grace and Rayment³, we consider that the key to climatic sensitivity of soil carbon is not total ecosystem respiration but decomposition rates (carbon loss per unit of fractionated carbon pool). The high respiration rates at the northern EUROFLUX sites¹⁴ are probably due to detritus accumulation under cool moist conditions, and not to temperature insensitivity of decomposition; even slow turnover of large stocks produces a lot of CO₂.

We agree with Giardina and Ryan that temperature effects on soil carbon dynamics may be overestimated in current biogeochemical models. However, feedback to global warming does not concern just temperature, but also includes its effect on soil water content and drainage, for example, and applies to all detrital carbon, whether on top of mineral soil or buried in peatlands and permafrost.

On the basis of the new results^{2,14}, Grace and Rayment³ suggest that the doomsday view of runaway global warming now seems unlikely. We believe that, on the contrary, the evidence remains in favour of a strong climatic control over storage of detrital carbon in terrestrial ecosystems.

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Giardina and Ryan reply — Davidson et al. question the validity of our conclusions on the grounds that differences in disturbance, high variability among sites, and the use of a single-pool model to estimate turnover

time (TT) obscured the effect of temperature on the decomposition of soil carbon.

In our comparison of incubated soils, disturbance associated with sieving and mixing probably increased decomposition rates. However, soil processing was similar across sites, and the resulting increase in carbon availability should have accentuated an effect of temperature on TT. In our *in situ* comparison, most tropical sites were pastures and all of the temperate sites were cropped. But these differences did not obscure an effect of temperature on TT. First, a subset of tropical sites had been continuously cultivated with sugar cane, which involves biannual mechanized ploughing, burning and fertilization. For sites that had been similarly disturbed after conversion from forest — that is, under intensive cultivation — TT tended to increase with mean annual temperature (MAT; $R^2 = 0.14$, $P = 0.07$, $n = 25$). Second, tropical pastures are more severely disturbed than Davidson et al. suggest — bulldozers may be used to level a site and remove stumps, and mechanized disking is often used to control weeds^{1,2}. Further, the effects of pasture conversion on carbon decomposition rates remain poorly understood².

There are two ways to test whether we missed significance because of high variability. First, how important is site-to-site variability? And second, given our sample size, how much variation must temperature explain in order to be significant? For the incubation comparison, variation in TT was low (coefficient of variation, 37%), and TT actually increased with temperature ($TT = 0.09 \times MAT + 6.2$). For the *in situ* comparison, time since conversion explained 34% of the variance in per cent carbon mass loss, a measure that assumes no model or age distribution of carbon in soil. With a sample size of 44 sites for the *in situ* comparison, MAT need only explain 8% of the variance to be significant at the 0.05 level. Given that MAT explained less than 1% of this variance, more than 1,000 sites would be needed to establish that there is a global relationship between temperature and soil carbon turnover.

We examined whether a single-pool model could obscure a temperature effect, and concluded that this is unlikely because our methods were robust^{1,3}, the tests were independent and well replicated, and neither comparison showed the negative trends between TT and temperature that would be expected from modelled patterns of carbon mass loss across latitude⁴. We agree with Davidson et al. that only a small

fraction of total soil carbon may be sensitive to temperature — this was our point.

Although the ¹⁴C approach preferred by Davidson et al. is useful for examining carbon turnover in soil, it has some methodological problems. For example, the ¹⁴C decay of interest begins at photosynthesis, not after incorporation into soil, as is now assumed^{1,5}. In forests, this lag could affect estimates of soil-carbon TT that are based on ¹⁴C because carbon can reside in living biomass and the litter layer for decades before being released to mineral soil. The ¹⁴C approach is also sensitive to contamination³ and has yielded much faster estimates of soil-carbon turnover than more direct methods¹.

Modelling terrestrial carbon storage depends on an accurate understanding of how temperature affects carbon TT in soil, but this effect cannot be inferred from large-scale relationships between soil-carbon content and MAT. Along gradients similar to those used by Jenny⁶ and cited by Davidson et al., plant primary production declines with increasing MAT, and it is this decline, not an increase in soil-carbon decomposition rate, that explains decreasing soil-carbon content with increasing temperature⁷. Similar findings have been reported⁸ for MAT gradients in northern Europe, and the gradient study cited by Davidson et al. actually reports⁹ longer carbon TT for tropical forest than for temperate forest. Despite the evidence against an exponential effect of temperature on soil carbon turnover, future *in situ* warming studies are needed to settle this issue.

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A. thaliana genome

By many criteria, plant life dominates Earth. More than 250,000 species of flowering plants decorate the world — and the entire biosphere depends on plants for food and oxygen. The sequencing and analysis of the first genome from the plant kingdom is therefore a scientific event of some importance. Add in the fact that plants and animals have independently solved the problems of multicellular existence, and the sequence can be rightly seen as a landmark. The last common ancestor of plants and animals existed over one-and-a-half billion years ago, so comparisons between the genomes of plants and other eukaryotes will provide fundamental insights into how life works.

The genome in question is that of *Arabidopsis thaliana*, a modest little flowering brassica, related to broccoli and cauliflower. *A. thaliana* is the “model” plant for research. Its small size, short life cycle and prodigious seed production make it an easy and inexpensive organism to propagate in the laboratory, and with a (relatively) small genome, of about 120 Mb, it is an ideal choice for sequencing. For comparison, the genomes of maize and wheat are 2,500 Mb and 16,000 Mb, respectively. *A. thaliana* lacks much of the repetitious DNA that riddles the genomes of higher plants, but it does contain a complete set of genes for controlling developmental patterns, metabolism, responses to environmental cues and disease resistance. Thus its genomic sequence provides a means for analysing gene function relevant to a range of plant species, including commercially important crops.

Although papers on *A. thaliana* appear weekly, fewer than 10% of the nearly 26,000 genes have been studied in experiments on gene function. The completion of the genome sequence will broaden and accelerate research. The *Arabidopsis* research community embraced a collegial spirit from its inception, openly sharing information and resources around the world. So it is fitting that the community celebrates the completion of the *A. thaliana* genome — four years ahead of schedule and well within budget — with today’s publication of the sequences of the plant’s remaining three chromosomes (the first two were published in the 16 December 1999 issue of *Nature*) and a comprehensive overview of the annotation and analysis of the genome sequence.

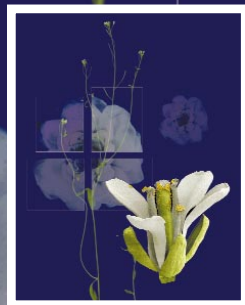
In an accompanying News and Views article, Virginia Walbot discusses how the genome sequence enhances the understanding of plant evolution and reveals both the striking conservation of genetic mechanisms underpinning cellular processes and the intriguing differences between plants and other eukaryotes. A News Feature covers other plant genome initiatives underway. The package is completed by a poster illustrating the impact of *A. thaliana* genomics on plant biology and beyond, and the whole of the plant’s genome is captured on a CD-ROM, available on request from *Nature*.

As with all genomics research published in *Nature*, the papers published today on *A. thaliana* (plus the two previously published papers) can be freely accessed on *Nature*’s Genome Gateway (<http://www.nature.com/genomics>), which provides a portal to the latest in genome research.

In 1777 the British botanist and apothecary William Curtis described *A. thaliana* in his *Flora Londinensis* as a plant of “no particular virtues or uses”. With today’s publication of its genome it can justifiably claim to have risen to among the most significant plants in the kingdom.

Carina Dennis Genetics Editor

Christopher Surridge Plant Sciences Editor



Cover illustrations

Foreground shows wild-type *A. thaliana*, background shows a mutant in which all floral organs are converted to petals. (Images courtesy of J. Berger, T. Laux & E. Meyerowitz.)



Next in the sequence: the rice genome project aims to be complete by 2004.

MARK EDWARDS/STILL PICTURES

Now for the hard ones

Arabidopsis was an obvious choice for the first plant genome project, but it will never feed the world. David Adam reports on efforts to harvest the genomes of rice and other important crop plants.

No plant scientist would deny that the complete genome sequence of the thale cress *Arabidopsis thaliana* is a landmark. The information it contains should give a boost to just about every area of plant science. But many agricultural researchers would not have made *Arabidopsis* their number-one choice. As one cereal biologist asks mischievously: "When are we going to sequence a real plant?"

Arabidopsis was tackled first because it is a popular model organism. Just as its rapid life cycle and small size made it ideal for laboratory studies, its relatively small genome recommended it for sequencing.

Insights from the *Arabidopsis* sequence are likely to boost crop science, but the genomics of crop plants is a much bigger challenge. Many have genomes larger than our own (see table, opposite), thanks to a tendency for plants to carry duplicate copies of large sections of DNA. Often all their chromosomes are duplicated several times over, a phenomenon known as polyploidy.

Nevertheless, the rice genome should be available within four years. And although researchers are divided over the need to sequence more crops once *Arabidopsis* and rice are in the bag, others may eventually follow. "It will take a lot of money and improved

technology, but I believe that the full genome of maize, at least, will be sequenced eventually," says Ed Coe, a maize researcher at the University of Missouri in Columbia.

Gigantic genomes

The genetic baggage of many plants is thought to be an evolutionary insurance against an unpredictable environment. As little as a quarter of a plant's genes may be essential for growth. But having multiple copies of genes increases the chance that a plant will possess a particular variant that will make the difference between, for example, surviving a drought or perishing.

This genetic extravagance makes plants difficult genomic targets, and not just because they have so much DNA to trawl through. The many stretches of identical 'junk' DNA throughout the genome can send sequencers round in circles as they try to stitch sequenced fragments together.

At about 400 million base pairs long, the rice genome is some four times larger than that of *Arabidopsis*, but still relatively small for a cereal crop. Its manageable size is one reason why the International Rice Genome Sequencing Project (IRGSP) was launched two years ago. More importantly, rice is a staple food for half of the world's population.

And although global rice production has doubled over the past 30 years — thanks largely to the introduction of new varieties — a better knowledge of its genetics will be needed to continue this progress.

The bulk of the rice genes discovered so far were identified using fragments of complementary DNA, produced from the messenger RNA strands made when a gene is expressed — a good way to spot important genes. Some 40,000 of these expressed sequence tags (ESTs) have been published for rice so far, and many more are held in the proprietary libraries of agribiotech companies.

But studying genomes using ESTs alone is like doing astronomy with the naked eye. This is why Japan's Ministry of Agriculture, Forestry and Fisheries (MAFF) took the lead in setting up the IRGSP to decode the plant's 12 chromosomes. Originally intending to finish the job within a decade, this date has since been brought forward.

"We are very optimistic that we can finish by the end of 2004," says Takuji Sasaki, leader of MAFF's rice genome research programme. The project is making good progress, he adds, but success still depends largely on the scale of future funding, particularly in the United States.

The commercial sector also has rice in its



Ear splitting: researchers are divided over whether the maize genome should be sequenced.

sights. In April 1999, Celera Genomics of Rockville, Maryland, caused a stir with its boast that it could sequence the entire rice genome in just six weeks. That project never got off the ground. "We never started it because the other organizations that we approached to cooperate all had their own programmes," says Bill Tucker, manager of business development and licensing at Celera's agricultural genomics division.

The multinationals Novartis and DuPont have built up their own rice DNA sequence databases, and the agribusiness giant Monsanto caught many plant researchers napping when it announced that it had completed a draft rice sequence earlier this year. The work was done under contract by a team led by Leroy Hood, then at the University of Washington in Seattle. Even more surprising was the company's pledge to turn all of its hard-won data over to the public IRGSP.

Six months later, however, there are doubts over the Monsanto sequence's accuracy and extent. "The quality is not so good in some places and we have to be very careful when using it," says Sasaki. This reflects the company's commercial interests, he suspects. Some gene-rich regions are thoroughly done, for example, but other areas are covered only briefly. "The draft should only be considered a scaffold to accelerate the sequencing of the rice genome," Hood says.

The draft should save the IRGSP — originally expected to cost US\$200 million — time and money. Monsanto also stands to benefit, as the public project will finish the company's work by improving accuracy and plugging gaps — the most time-consuming part of any sequencing project.

The finished segments will then be released to the open databases, but the deal between the IRGSP and Monsanto allows the

company to view the data as they are being accumulated. "Monsanto will learn a lot more about the rice genome by putting the information in the public's hands," says Benjamin Burr, an IRGSP member at the Brookhaven National Laboratory in New York state.

After the rice genome is finished, sequencers must decide whether to go after other cereal crops with larger genomes. Some experts believe it would be a waste of effort. Although different cereals have significantly different amounts of DNA, they share a common set of genes.

Wheat, rye, barley, maize, sorghum and millet all seem to have a similar genetic layout to rice — the genes are in much the same order but the larger genomes have more junk DNA between genes. This should mean that much of the information from the rice genome can be applied to other plants. "It would be stupid to sequence maize and wheat," argues Burr.

Against the grain

But others disagree. "Rice is a useful framework for studying other cereals, but it isn't perfect," says Rob Martienssen of the Cold Spring Harbor Laboratory on Long Island. He thinks that efforts to sequence maize will be under way within ten years — and by 2003, if current trends continue, advances in technology will make it cheaper to sequence maize than it is currently to do rice.

Whether full-blown sequencing takes off or not, work on cereal ESTs will continue. Monsanto, Novartis and AstraZeneca are all investigating ESTs in maize; and Pioneer Hi-Bred, a seed company owned by DuPont and based in Des Moines, Iowa, claims to have identified about 80% of the genes expressed in maize. Publicly funded researchers are also making progress. A public database of maize ESTs coordinated by Virginia Walbot at Stanford University in California, for instance, now has more than 70,000 entries.

While cereal scientists debate the necessity of sequencing crops other than rice, those working on brassicas such as oilseed rape (canola) could soon be reaping benefits from the *Arabidopsis* sequence, as the model organism is a member of the same family. The insights they gain will flow in large part from a public functional genomics project called *Arabidopsis* 2010. Its ambitious aim is to work out the function of each of the plant's 25,000 genes within a decade, and enter them into a database with information about the proteins they code for.

As the functional information piles up, researchers working on other crops will discover how much the results from *Arabidopsis* apply to their species. Russel Kohel of the US Department of Agriculture and Texas A&M University in College Station, for instance, is relying on comparisons with *Arabidopsis* for

his work on cotton. "We're not even considering sequencing," he says.

In the long run, many plant scientists would like to sequence at least one member of two other important plant groups: the legumes, which include peas, beans and other pulses; and the Solanaceae, which include potatoes and tomatoes. But for now, ESTs are the only game in town.

Randy Shoemaker at Iowa State University in Ames is striving to improve soya bean crops — funded in part by a cooperative of local farmers — and has banked over 130,000 publicly available ESTs. He aims to double this within 18 months, but does not expect the plant to be fully sequenced in the foreseeable future.

Meanwhile, plant genomics is spawning a series of public–corporate collaborations. DuPont is investing up to £15 million (US\$22 million) in work on wheat at the John Innes Centre in Norwich, and the centre has agreed a £50 million, ten-year collaboration with AstraZeneca. In France, several research institutes and companies joined forces in 1998 to launch a \$200 million initiative called Génoplante. And in 1999, Novartis signed a \$50 million agreement with the Department of Plant and Microbial Biology at the University of California, Berkeley.

Like Monsanto, these companies are realizing that raw sequence information may be of little use without the context that academic researchers can provide. "There has been a realization that sharing and collaboration offer a way forward," says Burr.

David Adam is a member of *Nature's* science writing team.

Web links:

- Arabidopsis Genome Initiative
- ♦ <http://www.arabidopsis.org/agi.html>
- International Rice Genome Sequencing Project
- ♦ <http://rgp.dna.affrc.go.jp/Seqcollab.html>
- Monsanto Rice Genome Project
- ♦ http://www.monsanto.com/monsanto/biotechnology/background_information/00Apr03_rice.html
- Arabidopsis 2010 Project
- ♦ <http://nasc.nott.ac.uk/garnet/2010.html>
- Maize EST database
- ♦ <http://www.zmdb.iastate.edu>

Species		Genome size (base pairs)
Brassicas		
Thale cress	<i>Arabidopsis thaliana</i>	1.0×10^8
Oilseed rape/canola	<i>Brassica napus</i>	1.2×10^9
Cereals		
Rice	<i>Oryza sativa</i>	4.2×10^8
Barley	<i>Hordeum vulgare</i>	4.8×10^8
Wheat	<i>Triticum aestivum</i>	1.6×10^{10}
Maize/corn	<i>Zea mays</i>	2.5×10^9
Legumes		
Garden pea	<i>Pisum sativum</i>	4.1×10^8
Soya bean	<i>Glycine max</i>	1.1×10^9
Solanaceae		
Potato	<i>Solanum tuberosum</i>	1.8×10^9
Tomato	<i>Lycopersicon esculentum</i>	1.0×10^9
Human	<i>Homo sapiens</i>	3.2×10^9

A green chapter in the book of life

Virginia Walbot

The sequencing of an entire plant genome is now complete. The information it contains provides an unparalleled resource for understanding the evolution of flowering plants and the genetics of crop plants.

Flowering plants are the most remarkable success story in recent evolution¹. They arose only about 200 million years ago. But today there are roughly 250,000 flowering species, and they dominate nearly every terrestrial, aquatic and estuarine ecosystem. Humans rely on flowering plants for calories, essential amino acids, vitamins and thousands of chemicals and pharmaceutical drugs. The diversity of form and chemistry among these plants is astounding. But, given their relatively recent diversification, one would expect most genes identified in one species to have counterparts in all the others. Features that distinguish roses from lilies, and palms from plum trees, will be explained by alterations in gene regulation or in the activity of proteins — easily identifiable against a backdrop of similar gene types and sequences. So all plant biologists will benefit immediately from the publication, on pages 796–826 of this issue^{2–5}, of the sequences of the final three of the five chromosomes of thale cress, *Arabidopsis thaliana*. The other two were published a year ago^{6,7}.

Achieving an objective set in 1996, the *Arabidopsis* sequencing consortium has sequenced 118.7 million base pairs of this

plant's nuclear genome^{2–4,6,7}. Improvements in technology mean that this is the most accurate eukaryotic genome sequence obtained so far⁵ — the others being those of the yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*. The *Arabidopsis* sequence also has the fewest gaps in the gene-rich segments of the chromosomes⁵. Substantial sequence is also available for the five centromeres — gene-poor structural DNA needed for the pairing and movement of chromosomes during cell division^{8,9}.

One might think that crop plants would be more immediately useful to study than this tiny weed. But such plants are themselves large (Box 1), and their genomes are also often large and difficult to manipulate. The rapid rise of *Arabidopsis* as the model experimental flowering plant was based on the argument that it had but one copy of each gene and less than 10% repetitive DNA¹⁰. With the complete *Arabidopsis* genome sequence in hand, however, the major surprise is the amount of genetic redundancy⁵. About 26,000 genes have been identified in the sequence. But, astonishingly, at least 70% of the genome has been duplicated. In all,

there are fewer than 15,000 different genes — a figure that could shrink again as researchers recognize duplicated genes whose sequences have diverged further.

In other flowering plants, two factors contribute to gene redundancy: polyploidization (duplication of the entire set of chromosomes) and local gene duplications (copying of individual genes within chromosomes). Both processes occurred in the history of *Arabidopsis*⁵. Two polyploidization events — dated to about 180 million and 112 million years ago — explain duplications of suites of genes on two or more chromosomes. And some 17% of the genes are represented in local duplications. Gene loss and chromosome rearrangements then resulted in the small genome and five chromosomes of *Arabidopsis* today. In contrast, its diverse brassica crop relatives — cabbage, cauliflower and so on (Fig. 1) — shared a common ancestor with *Arabidopsis* about 12 million to 19 million years ago, but have increased in genome size as a result of further polyploidization events.

Arabidopsis genes are compact, typically containing several coding regions (exons) of about 250 base pairs each, punctuated by short non-coding regions (introns). The genes are closely spaced, about 4.6 kilobases apart, indicating that their regulatory regions are also short. Many animal genes, by contrast, contain dozens of exons, and have regulatory regions of 10 kilobases or larger. The small genes of *Arabidopsis* may have helped it to tolerate extensive genomic reorganization — the smaller the gene, the less likely that it will be disrupted. Indeed, plants with much larger genomes also have compact genes. However, the distance between genes in such plants is one or two orders of magnitude greater than in *Arabidopsis*, as a result of the insertion of mobile DNA elements¹¹. In *Arabidopsis*, such mobile elements are confined mainly to the gene-poor regions near centromeres⁹.

Despite the apparent gene duplication, geneticists have identified thousands of mutations (in maize, tomato, *Arabidopsis* and bread wheat) that produce visible defects in the plant but occur in only one of the duplicated genes. If both copies had the same function, one would expect mutation in just one of the genes to be compensated by the other. So, many duplicated genes in these species have unique roles. Mutations in regulatory regions can yield duplicated genes that

Box 1 Big advantages of a little weed

One thousand *Arabidopsis* plants could grow and reproduce in the space of this page. This small size is advantageous to researchers studying plant biology. Agricultural species are hundreds (in the case of soybean or maize), if not tens of thousands (in the case of apple trees), of times larger than *Arabidopsis*. But most developmental and physiological processes in *Arabidopsis*, as well as the genes controlling them, will have counterparts in crop plants.

Using *Arabidopsis*, researchers can manipulate light, soil and nutrients to study the effects on organ development, physiology and

seed production. They can introduce viral, bacterial and fungal pathogens, as well as insect pests, into these miniature laboratory fields to assess damage. Replicate experiments can compare the performance of wild-type and mutant plants, identifying plant genes crucial to vegetative growth and reproductive success. Such controlled conditions are impossible with agricultural species. But with the *Arabidopsis* sequence in hand, finding a crop gene involved in any of these processes is much simpler than before. The exceptions are the genes underlying unusual features, such as the ability of legumes to form nitrogen-fixing organs containing symbiotic

bacteria, and C4 photosynthesis in sugar cane and maize.

Improving crop yields also requires knowing how plants scale up their body plan. Which genes determine the weight of a tomato or the size of grains? In all of this, we should remember the influence of humans. We started domesticating plants only 8,000 years ago, from some rather unpromising wild progenitors. Selection of traits that improve our diet and make harvesting easier have changed the pea-sized wild tomato into the modern giant, and the bone-hard teosinte seeds into the large, soft, modern maize. Studies of *Arabidopsis* will help to determine the genetic basis for these changes. **V.W.**

are expressed differently during development or in response to environmental changes. Mutations in the coding regions can result in subtly different proteins. Starting with the raw material provided by gene duplications, flowering-plant evolution has built on both types of mutational divergence to produce new, species-specific plant structures and chemistries.

The exons in *Arabidopsis* are richer in guanosine and cytosine bases (44%) than are the introns (32%) — a distinctive feature of plant genes. When a gene is transcribed, a pre-messenger RNA is produced first; the introns are then removed to produce functional messenger RNA. Differences in base composition may contribute to the accuracy of this process through large fluctuations in daily and seasonal temperatures¹².

The number of different *Arabidopsis* genes (less than 15,000) is only slightly larger than the 13,601 predicted for *D. melanogaster* and fewer than the 18,424 for *C. elegans*¹³. Most of the *Arabidopsis* genes have counterparts in these animal species, indicating the common ancestry of plants and animals. In all three genomes, the number of genes is similar to the number of words in a paperback dictionary. And, just as a huge number of books can be written by linking these words together in different ways, so the diversity of life depends to a certain extent on how the genes are coupled together into pathways, with selection resulting in shades of meaning that denote species-specific function. During 1.6 billion years of divergence of plants and animals, the larger context and functional meaning of conserved biochemical players, such as transcription factors and protein kinases, have diverged substantially.

For example, several classes of gene are particularly numerous in *Arabidopsis*. There are many genes that encode water-transporting channels, for example, and the genome encodes ten times more peptide-hormone transporters than are found in animal genomes. There are hundreds of putative receptor-like protein kinases, but many components of animal signal-transduction pathways are not present. More than 420 *Arabidopsis* genes may be involved in synthesizing or modifying cell walls⁵, which animals lack. Nearly 25% of the nuclear genes contain signal sequences — address labels that are predicted to direct the encoded proteins into organelles such as chloroplasts or mitochondria. In animals, fewer than 5% of the proteins include signal sequences that will

direct them to mitochondria. This is perhaps not surprising, as the plant organelles between them carry out many more metabolic reactions than occur in the organelles of animals and fungi.

These metabolic transactions between the inorganic and organic world make animal and fungal life possible. Plants use solar energy to convert carbon dioxide into sugars, carbohydrate polymers and lipids. They reduce nitrate and sulphate ions to synthesize amino acids. They make every vitamin and enzyme cofactor, and are the primary conduit for concentrating and hence making available phosphate, iron, zinc, magnesium, potassium and all the other mineral nutrients in animal diets. These metabolic capabilities are shared with photosynthetic bacteria, and indeed *Arabidopsis* and the cyanobacterium *Synechocystis*¹⁴ have many of the same genes. So it is not surprising that metabolic and biosynthetic genes are much more numerous in *Arabidopsis* than in the other available eukaryotic genomes, comprising at least 10% of the genome.

Flowering plants are also estimated to synthesize at least 100,000 secondary compounds — those that are not found in animals, and are not essential to the life of the plant — many of which are species- or genus-specific. Diversification of chemistry, readily measured by our taste buds, supplies dyes, flavours, fragrances and most of the therapeutic drugs that we use. Although no flowering plant synthesizes all of these astonishingly diverse products, the repertoire of *Arabidopsis* genes contains the information to synthesize the archetypal precursors of secondary products. Chemical differences between plant species are reflected mainly in the modifications of these core molecules.

Flowering plants use chemicals to attract pollinators and repel pests. This coevolutionary chemical arms race stimulates the rapid proliferation of species — for example, 6,000 species of hispine leaf beetles feed on the ginger family alone¹⁵. Genes involved in detoxification are common in *Arabidopsis*, probably reflecting its ability to store harmful compounds.

With the genome in hand, the next challenge will be to investigate the roles of individual *Arabidopsis* proteins. And studies of other flowering plants will pinpoint those genes responsible for structural and biochemical diversity between species, as well as the likely path of evolutionary gene change that produced this diversity. Progress



Figure 1 Diversity in the brassicas. Relatives of *Arabidopsis* (above) include vegetables such as cabbages, spices such as mustard, and oil-producing crops including rapeseed (bottom left) and canola. Selection by humans over several thousand years has produced brussels sprouts, cauliflower and cabbage (left, from top), as well as other vegetables, from just one species, *Brassica oleracea*.

towards these goals will be quickened by the US National Science Foundation's \$150 million investment in the first three years of the Plant Genome Award Program¹⁶, and the newly announced 2010 Program in Functional Genomes¹⁷. Similarly large investments in Europe and Asia will speed our understanding of this highly successful branch of the plant kingdom on which we are all so heavily dependent.

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Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*

The Arabidopsis Genome Initiative*

* Authorship of this paper should be cited as 'The Arabidopsis Genome Initiative'. A full list of contributors appears at the end of this paper

The flowering plant *Arabidopsis thaliana* is an important model system for identifying genes and determining their functions. Here we report the analysis of the genomic sequence of *Arabidopsis*. The sequenced regions cover 115.4 megabases of the 125-megabase genome and extend into centromeric regions. The evolution of *Arabidopsis* involved a whole-genome duplication, followed by subsequent gene loss and extensive local gene duplications, giving rise to a dynamic genome enriched by lateral gene transfer from a cyanobacterial-like ancestor of the plastid. The genome contains 25,498 genes encoding proteins from 11,000 families, similar to the functional diversity of *Drosophila* and *Caenorhabditis elegans*—the other sequenced multicellular eukaryotes. *Arabidopsis* has many families of new proteins but also lacks several common protein families, indicating that the sets of common proteins have undergone differential expansion and contraction in the three multicellular eukaryotes. This is the first complete genome sequence of a plant and provides the foundations for more comprehensive comparison of conserved processes in all eukaryotes, identifying a wide range of plant-specific gene functions and establishing rapid systematic ways to identify genes for crop improvement.

The plant and animal kingdoms evolved independently from unicellular eukaryotes and represent highly contrasting life forms. The genome sequences of *C. elegans*¹ and *Drosophila*² reveal that metazoans share a great deal of genetic information required for developmental and physiological processes, but these genome sequences represent a limited survey of multicellular organisms. Flowering plants have unique organizational and physiological properties in addition to ancestral features conserved between plants and animals. The genome sequence of a plant provides a means for understanding the genetic basis of differences between plants and other eukaryotes, and provides the foundation for detailed functional characterization of plant genes.

Arabidopsis thaliana has many advantages for genome analysis, including a short generation time, small size, large number of offspring, and a relatively small nuclear genome. These advantages promoted the growth of a scientific community that has investigated the biological processes of *Arabidopsis* and has characterized many genes³. To support these activities, an international collaboration (the Arabidopsis Genome Initiative, AGI) began sequencing the genome in 1996. The sequences of chromosomes 2 and 4 have been reported^{4,5}, and the accompanying Letters describe the sequences of chromosomes 1 (ref. 6), 3 (ref. 7) and 5 (ref. 8).

Here we report analysis of the completed *Arabidopsis* genome sequence, including annotation of predicted genes and assignment of functional categories. We also describe chromosome dynamics and architecture, the distribution of transposable elements and other repeats, the extent of lateral gene transfer from organelles, and the comparison of the genome sequence and structure to that of other *Arabidopsis* accessions (distinctive lines maintained by single-seed descent) and plant species. This report is the summation of work by experts interested in many biological processes selected to illuminate plant-specific functions including defence, photomorphogenesis, gene regulation, development, metabolism, transport and DNA repair.

The identification of many new members of receptor families, cellular components for plant-specific functions, genes of bacterial origin whose functions are now integrated with typical eukaryotic components, independent evolution of several families of transcription factors, and suggestions of as yet uncharacterized metabolic pathways are a few more highlights of this work. The implications of these discoveries are not only relevant for plant

biologists, but will also affect agricultural science, evolutionary biology, bioinformatics, combinatorial chemistry, functional and comparative genomics, and molecular medicine.

Overview of sequencing strategy

We used large-insert bacterial artificial chromosome (BAC), phage (P1) and transformation-competent artificial chromosome (TAC) libraries^{9–12} as the primary substrates for sequencing. Early stages of genome sequencing used 79 cosmid clones. Physical maps of the genome of accession Columbia were assembled by restriction fragment 'fingerprint' analysis of BAC clones¹³, by hybridization¹⁴ or polymerase chain reaction (PCR)¹⁵ of sequence-tagged sites and by hybridization and Southern blotting¹⁶. The resulting maps were integrated (<http://nucleus.cshl.org/arabmaps/>) with the genetic map and provided a foundation for assembling sets of contigs into sequence-ready tiling paths. End sequence (http://www.tigr.org/tldb/at/abe/bac_end_search.html) of 47,788 BAC clones was used to extend contigs from BACS anchored by marker content and to integrate contigs.

Ten contigs representing the chromosome arms and centromeric heterochromatin were assembled from 1,569 BAC, TAC, cosmid and P1 clones (average insert size 100 kilobases (kb)). Twenty-two PCR products were amplified directly from genomic DNA and sequenced to link regions not covered by cloned DNA or to optimize the minimal tiling path. Telomere sequence was obtained from specific yeast artificial chromosome (YAC) and phage clones, and from inverse polymerase chain reaction (IPCR) products derived from genomic DNA. Clone fingerprints, together with BAC end sequences, were generally adequate for selection of clones for sequencing over most of the genome. In the centromeric regions, these physical mapping methods were supplemented with genetic mapping to identify contig positions and orientation¹⁷.

Selected clones were sequenced on both strands and assembled using standard techniques. Comparison of independently derived sequence of overlapping regions and independent reassembly sequenced clones revealed accuracy rates between 99.99 and 99.999%. Over half of the sequence differences were between genomic and BAC clone sequence. All available sequenced genetic markers were integrated into sequence assemblies to verify sequence contigs^{4–8}. The total length of sequenced regions, which extend from either the telomeres or ribosomal DNA repeats to the 180-base-pair

(bp) centromeric repeats, is 115,409,949 bp (Table 1). Estimates of the unsequenced centromeric and rDNA repeat regions measure roughly 10 megabases (Mb), yielding a genome size of about 125 Mb, in the range of the 50–150 Mb haploid content estimated by different methods¹⁸. In general, features such as gene density, expression levels and repeat distribution are very consistent across the five chromosomes (Fig. 1), and these are described in detail in reports on individual chromosomes^{4–8} and in the analysis of centromere, telomere and rDNA sequences.

We used tRNAscan-SE 1.21 (ref. 19) and manual inspection to identify 589 cytoplasmic transfer RNAs, 27 organelle-derived tRNAs and 13 pseudogenes—more than in any other genome sequenced to date. All 46 tRNA families needed to decode all possible 61 codons were found, defining the completeness of the functional set. Several highly amplified families of tRNAs were found on the same strand⁶; excluding these, each amino acid is decoded by 10–41 tRNAs.

The spliceosomal RNAs (U1, U2, U4, U5, U6) have all been experimentally identified in *Arabidopsis*. The previously identified

sequences for all RNAs were found in the genome, except for U5 where the most similar counterpart was 92% identical. Between 10 and 16 copies of each small nuclear RNA (snRNA) were found across all chromosomes, dispersed as singletons or in small groups.

The small nucleolar RNAs (snoRNAs) consist of two subfamilies, the C/D box snoRNAs, which includes 36 *Arabidopsis* genes, and the H/ACA box snoRNAs, for which no members have been identified in *Arabidopsis*. U3 is the most numerous of the C/D box snoRNAs, with eight copies found in the genome. We identified forty-five additional C/D box snoRNAs using software (www.rna.wustl.edu/snoRNAdb/) that detects snoRNAs that guide ribose methylation of ribosomal RNA.

A combination of algorithms, all optimized with parameters based on known *Arabidopsis* gene structures, was used to define gene structure. We used similarities to known protein and expressed sequence tag (EST) sequence to refine gene models. Eighty per cent of the gene structures predicted by the three centres involved were completely consistent, 93% of ESTs matched gene models, and less than 1% of ESTs matched predicted non-coding regions, indicating

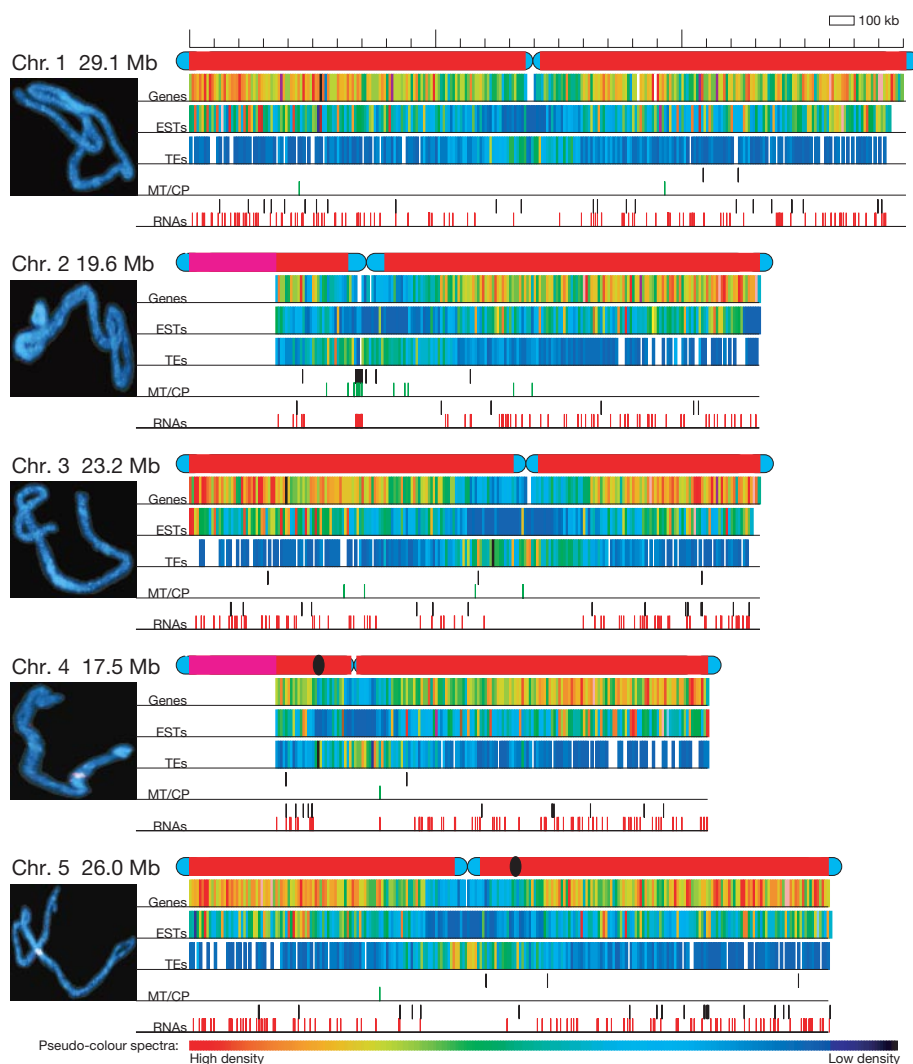


Figure 1 Representation of the *Arabidopsis* chromosomes. Each chromosome is represented as a coloured bar. Sequenced portions are red, telomeric and centromeric regions are light blue, heterochromatic knobs are shown black and the rDNA repeat regions are magenta. The unsequenced telomeres 2N and 4N are depicted with dashed lines. Telomeres are not drawn to scale. Images of DAPI-stained chromosomes were kindly supplied by P. Fransz. The frequency of features was given pseudo-colour assignments, from red (high density) to deep blue (low density). Gene density ('Genes')

ranged from 38 per 100 kb to 1 gene per 100 kb; expressed sequence tag matches ('ESTs') ranged from more than 200 per 100 kb to 1 per 100 kb. Transposable element densities ('TEs') ranged from 33 per 100 kb to 1 per 100 kb. Mitochondrial and chloroplast insertions ('MT/CP') were assigned black and green tick marks, respectively. Transfer RNAs and small nucleolar RNAs ('RNAs') were assigned black and red ticks marks, respectively.

that most potential genes were identified. The sensitivity and selectivity of the gene prediction software used in this report has been comprehensively and independently assessed²⁰.

The 25,498 genes predicted (Table 1) is the largest gene set published to date: *C. elegans*¹ has 19,099 genes and *Drosophila*² 13,601 genes. *Arabidopsis* and *C. elegans* have similar gene density, whereas *Drosophila* has a lower gene density; *Arabidopsis* also has a significantly greater extent of tandem gene duplications and segmental duplications, which may account for its larger gene set.

The rDNA repeat regions on chromosomes 2 and 4 were not sequenced because of their known repetitive structure and content. The centromeric regions are not completely sequenced owing to large blocks of monotonic repeats such as 5S rDNA and 180-bp repeats. The sequence continues to be extended further into centromeric and other regions of complex sequence.

Characterization of the coding regions

To assess the similarities and differences of the *Arabidopsis* gene complement compared with other sequenced eukaryotic genomes, we assigned functional categories to the complete set of *Arabidopsis* genes. For chromosome 4 genes and the yeast genome, predicted functions were previously manually assigned^{5,21}. All other predicted proteins were automatically assigned to these functional categories²², assuming that conserved sequences reflect common functional relationships.

The functions of 69% of the genes were classified according to sequence similarity to proteins of known function in all organisms; only 9% of the genes have been characterized experimentally (Fig. 2a). Generally similar proportions of gene products were predicted to be targeted to the secretory pathway and mitochondria in *Arabidopsis* and yeast, and up to 14% of the gene products are

Table 1 Summary statistics of the *Arabidopsis* genome

Feature	Value											
(a) The DNA molecules												
	Chr. 1		Chr. 2		Chr. 3		Chr. 4		Chr. 5		Σ	
Length (bp)	29,105,111		19,646,945		23,172,617		17,549,867		25,953,409		115,409,949	
Top arm (bp)	14,449,213		3,607,091		13,590,268		3,052,108		11,132,192			
Bottom arm (bp)	14,655,898		16,039,854		9,582,349		14,497,759		14,803,217			
Base composition (%GC)												
Overall	33.4		35.5		35.4		35.5		34.5			
Coding	44.0		44.0		44.3		44.1		44.1			
Non-coding	32.4		32.9		33.0		32.8		32.5			
Number of genes	6,543		4,036		5,220		3,825		5,874		25,498	
Gene density (kb per gene)	4.0		4.9		4.5		4.6		4.4			
Average gene length (bp)	2,078		1,949		1,925		2,138		1,974			
Average peptide length (bp)	446		421		424		448		429			
Exons												
Number	35,482		19,631		26,570		20,073		31,226		13,2982	
Total length (bp)	8,772,559		5,100,288		6,654,507		5,150,883		7,571,013		33,249,250	
Average per gene	5.4		4.9		5.1		5.2		5.3			
Average size (bp)	247		259		250		256		242			
Introns												
Number	28,939		15,595		21,350		16,248		25,352		107,484	
Total length (bp)	4,828,766		2,768,430		3,397,531		3,030,649		4,030,045		18,055,421	
Average size (bp)	168		177		159		186		159			
Number of genes with ESTs (%)	60.8		56.9		59.8		61.4		61.4			
Number of ESTs	30,522		14,989		20,732		16,605		22,885		105,733	
(b) The proteome												
Classification/function												
Total proteins	6,543		4,036		5,220		3,825		5,874		25,498	
With INTERPRO domains	4,194		1,205		2,989		1,545		3,136		13,069	
Genes containing at least one TM domain	64.1%		29.9%		57.8%		40.4%		53.4%		51.3%	
Genes containing at least one SCOP domain	2,334		1,322		1,615		1,402		1,940		8,613	
With putative signal peptides	35.7%		32.8%		30.9%		36.7%		33.0%		33.8%	
Secretory pathway	2,513		1,424		1,664		1,304		2,121		9,026	
>0.95 specificity	38.4%		35.3%		31.9%		34.1%		36.1%		35.4%	
Chloroplast	1,242		675		877		659		1,014		4,467	
>0.95 specificity	19.0%		16.7%		17.0%		17.2%		17.3%		17.6%	
Mitochondria	1,146		632		813		632		964		4,167	
>0.95 specificity	17.5%		15.7%		15.7%		16.5%		16.4%		16.4%	
>0.95 specificity	866		535		754		532		887		3,574	
>0.95 specificity	13.2%		13.2%		14.6%		13.9%		15.1%		14.0%	
>0.95 specificity	602		290		420		298		475		2,085	
>0.95 specificity	9.2%		7.2%		8.1%		7.8%		8.1%		8.2%	
>0.95 specificity	901		425		554		390		627		2,897	
>0.95 specificity	13.8%		10.5%		10.7%		10.2%		10.7%		11.4%	
>0.95 specificity	113		49		63		59		65		349	
>0.95 specificity	1.7%		1.2%		1.2%		1.5%		1.1%		1.4%	
Functional classification												
Cellular metabolism	1,188		620		745		588		868		4,009	
Transcription	22.7%		23.3%		22.8%		22.9%		21.1%		22.5%	
Plant defence	880		474		566		335		763		3,018	
Signalling	16.8%		17.8%		17.3%		13.1%		18.6%		16.9%	
Growth	640		276		354		295		490		2,055	
Protein fate	12.2%		10.4%		10.8%		11.5%		11.9%		11.5%	
Intracellular transport	573		296		356		210		420		1,855	
Transport	11.0%		11.1%		10.9%		8.2%		10.2%		10.4%	
Protein synthesis	542		263		357		448		469		2,079	
	10.4%		9.9%		10.9%		17.5%		11.4%		11.7%	
	520		273		314		264		395		1,766	
	9.9%		10.2%		9.6%		10.3%		9.6%		9.9%	
	435		214		269		220		334		1,472	
	8.3%		8.9%		8.2%		8.6%		8.1%		8.3%	
	236		139		155		113		206		849	
	4.5%		5.2%		4.7%		4.4%		5.0%		4.8%	
	216		111		148		90		165		730	
	4.1%		4.2%		4.5%		3.5%		4.0%		4.1%	
Total	5,230		2,666		3,264		2,563		4,110		17,833	

The features of *Arabidopsis* chromosomes 1–5 and the complete nuclear genome are listed. Specialized searches used the following programs and databases: INTERPRO²³; transmembrane (TM) domains by ALOM2 (unpublished); SCOP domain database²¹; functional classification by the PEDANT analysis system²². Signal peptide prediction (secretory pathway, targeted to chloroplast or mitochondria) was performed using TargetP²² and <http://www.cbs.dtu.dk/services/TargetP/>.

* Default value.

likely to be targeted to the chloroplast (Table 1). The significant proportion of genes with predicted functions involved in metabolism, gene regulation and defence is consistent with previous analyses⁵. Roughly 30% of the 25,498 predicted gene products, (Fig. 2a), comprising both plant-specific proteins and proteins with similarity to genes of unknown function from other organisms, could not be assigned to functional categories.

To compare the functional categories in more detail, we compared data from the complete genomes of *Escherichia coli*²³, *Synechocystis* sp.²⁴, *Saccharomyces cerevisiae*²¹, *C. elegans*¹ and *Drosophila*², and a non-redundant protein set of *Homo sapiens*, with the *Arabidopsis* genome data (Fig. 2b), using a stringent BLASTP threshold value of $E < 10^{-30}$. The proportion of *Arabidopsis* proteins having related counterparts in eukaryotic genomes varies by a factor of 2 to 3 depending on the functional category. Only 8–23% of *Arabidopsis* proteins involved in transcription have related genes in other eukaryotic genomes, reflecting the independent evolution of many plant transcription factors. In contrast, 48–60% of genes involved in protein synthesis have counterparts in the other eukaryotic genomes, reflecting highly

conserved gene functions. The relatively high proportion of matches between *Arabidopsis* and bacterial proteins in the categories 'metabolism' and 'energy' reflects both the acquisition of bacterial genes from the ancestor of the plastid and high conservation of sequences across all species. Finally, a comparison between unicellular and multicellular eukaryotes indicates that *Arabidopsis* genes involved in cellular communication and signal transduction have more counterparts in multicellular eukaryotes than in yeast, reflecting the need for sets of genes for communication in multicellular organisms.

Pronounced redundancy in the *Arabidopsis* genome is evident in segmental duplications and tandem arrays, and many other genes with high levels of sequence conservation are also scattered over the genome. Sequence similarity exceeding a BLASTP value $E < 10^{-20}$ and extending over at least 80% of the protein length were used as parameters to identify protein families (Table 2). A total of 11,601 protein types were identified. Thirty-five per cent of the predicted proteins are unique in the genome, and the proportion of proteins belonging to families of more than five members is substantially higher in *Arabidopsis* (37.4%) than in *Drosophila* (12.1%) or

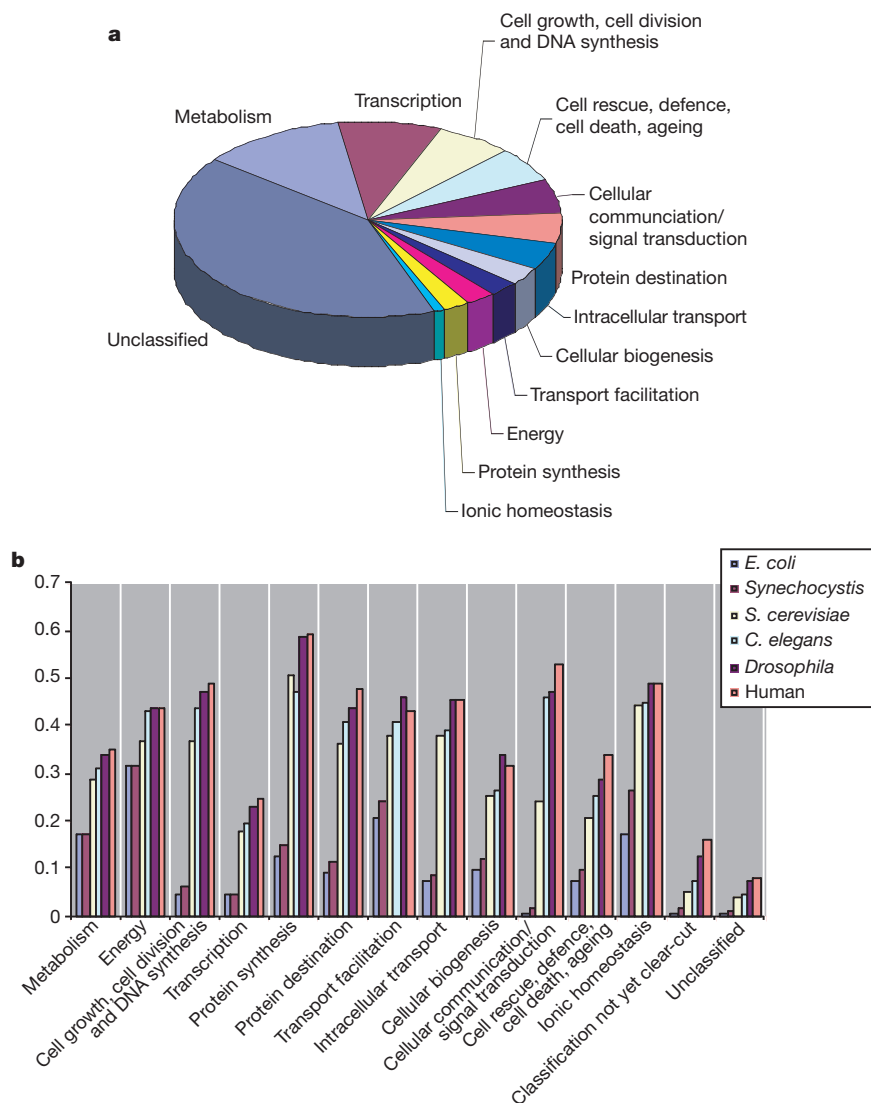


Figure 2 Functional analysis of *Arabidopsis* genes. **a**, Proportion of predicted *Arabidopsis* genes in different functional categories. **b**, Comparison of functional categories between organisms. Subsets of the *Arabidopsis* proteome containing all proteins that fall into a common functional class were assembled. Each subset was searched against the complete set of translations from *Escherichia coli*, *Synechocystis* sp. PCC6803,

Saccharomyces cerevisiae, *Drosophila*, *C. elegans* and a *Homo sapiens* non-redundant protein database. The percentage of *Arabidopsis* proteins in a particular subset that had a BLASTP match with $E \leq 10^{-30}$ to the respective reference genome is shown. This reflects the measure of sequence conservation of proteins within this particular functional category between *Arabidopsis* and the respective reference genome. y axis, 0.1 = 10%.

Table 2 Proportion of genes in different organisms present as either singletons or in paralogous families

	No of singletons and distinct gene families	Unique	Gene families containing				
			2 members	3 members	4 members	5 members	>5 members
<i>H. influenzae</i>	1,587	88.8%	6.8%	2.3%	0.7%	0.0%	1.4%
<i>S. cerevisiae</i>	5,105	71.4%	13.8%	3.5%	2.2%	0.7%	8.4%
<i>D. melanogaster</i>	10,736	72.5%	8.5%	3.4%	1.9%	1.6%	12.1%
<i>C. elegans</i>	14,177	55.2%	12.0%	4.5%	2.7%	1.6%	24.0%
<i>Arabidopsis</i>	11,601	35.0%	12.5%	7.0%	4.4%	3.6%	37.4%

The number of genes in the genomes of *Haemophilus influenzae*, *S. cerevisiae*, *Drosophila*, *C. elegans* and *Arabidopsis* that are present either as singletons or in gene families with two or more members are listed. To be grouped in a gene family, two genes had to show similarity exceeding a BLASTP value $E < 10^{-20}$ and a FASTA alignment over at least 80% of the protein length. In column 1, the number of genes that are unique plus the number of gene families are listed. Columns 2 to 6 give the percentage of genes present as singletons or in gene families of n members.

C. elegans (24.0%). The absolute number of *Arabidopsis* gene families and singletons (types) is in the same range as the other multicellular eukaryotes, indicating that a proteome of 11,000–15,000 types is sufficient for a wide diversity of multicellular life. The proportion of gene families with more than two members is considerably more pronounced in *Arabidopsis* than in other eukaryotes (Fig. 3). As segmental duplication is responsible for 6,303 gene duplications (see below), the extent of tandem gene duplications accounts for a significant proportion of the increased family size. These features of the *Arabidopsis*, and presumably other plant genomes, may indicate more relaxed constraints on genome size in plants, or a more prominent role of unequal crossing over to generate new gene copies.

Conserved protein domains revealed more informative differences through INTERPRO²⁵ analysis of the predicted gene products from *Arabidopsis*, *S. cerevisiae*, *C. elegans* and *Drosophila*. Statistically over-represented domains, and those that are absent from the *Arabidopsis* genome, indicate domains that may have been gained or lost during the evolution of plants (Supplementary Information Table 1). Proteins containing the Pro-Pro-Arg repeat, which is involved in RNA stabilization and RNA processing, are over-represented as compared to yeast, fly and worm; 400 proteins containing this signature were detected in *Arabidopsis* compared with only 10 in total in yeast, *Drosophila* and *C. elegans*. Protein kinases and associated domains, 169 proteins containing a disease resistance protein signature, and the Toll/IL-1R (TIR) domain, a component of pathogen recognition molecules²⁶, are also relatively abundant. This suggests that pathways transducing signals in response to pathogens and diverse environmental cues are more abundant in plants than in other organisms.

The RING zinc finger domain is relatively over-represented in *Arabidopsis* compared with yeast, *Drosophila* and *C. elegans*, whereas the F-box domain is over-represented as compared with yeast and *Drosophila* only. These domains are involved in targeting proteins to the proteasome²⁷ and ubiquitylation²⁸ pathways of protein degradation, respectively. In plants many processes such as hormone and defence responses, light signalling, and circadian rhythms and pattern formation use F-box function to direct negative regulators

to the ubiquitin degradation pathway. This mode of regulation appears to be more prevalent in plants and may account for a higher representation of the F box than in *Drosophila* and for the over-representation of the ubiquitin domain in the *Arabidopsis* genome. RING finger domain proteins in general have a role in ubiquitin protein ligases, indicating that proteasome-mediated degradation is a more widespread mode of regulation in plants than in other kingdoms.

Most functions identified by protein domains are conserved in similar proportions in the *Arabidopsis*, *S. cerevisiae*, *Drosophila* and *C. elegans* genomes, pointing to many ubiquitous eukaryotic pathways. These are illustrated by comparing the list of human disease genes²⁹ to the complete *Arabidopsis* gene set using BLASTP. Out of 289 human disease genes, 139 (48%) had hits in *Arabidopsis* using a BLASTP threshold $E < 10^{-10}$. Sixty-nine (24%) exceeded an $E < 10^{-40}$ threshold, and 26 (9.3%) had scores better than $E < 10^{-100}$ (Table 3). There are at least 17 human disease genes more similar to *Arabidopsis* genes than yeast, *Drosophila* or *C. elegans* genes (Table 3).

This analysis shows that, although numerous families of proteins are shared between all eukaryotes, plants contain roughly 150 unique protein families. These include transcription factors, structural proteins, enzymes and proteins of unknown function. Members of the families of genes common to all eukaryotes have undergone substantial increases or decreases in their size in *Arabidopsis*. Finally, the transfer of a relatively small number of cyanobacteria-related genes from a putative endosymbiotic ancestor of the plastid has added to the diversity of protein structures found in plants.

Genome organization and duplication

The *Arabidopsis* genome sequence provides a complete view of chromosomal organization and clues to its evolutionary history. Gene families organized in tandem arrays of two or more units have been described in *C. elegans*¹ and *Drosophila*². Analysis of the *Arabidopsis* genome revealed 1,528 tandem arrays containing 4,140 individual genes, with arrays ranging up to 23 adjacent members (Fig. 3). Thus 17% of all genes of *Arabidopsis* are arranged in tandem arrays.

Large segmental duplications were identified either by directly aligning chromosomal sequences or by aligning proteins and searching for tracts of conserved gene order. All five chromosomes were aligned to each other in both orientations using MUMmer³⁰, and the results were filtered to identify all segments at least 1,000 bp in length with at least 50% identity (Supplementary Information Fig. 1). These revealed 24 large duplicated segments of 100 kb or larger, comprising 65.6 Mb or 58% of the genome. The only duplicated segment in the centromeric regions was a 375-kb segment on chromosome 4. Many duplications appear to have undergone further shuffling, such as local inversions after the duplication event.

We used TBLASTX³ to identify collinear clusters of genes residing in large duplicated chromosomal segments. The duplicated regions encompass 67.9 Mb, 60% of the genome, slightly more than was

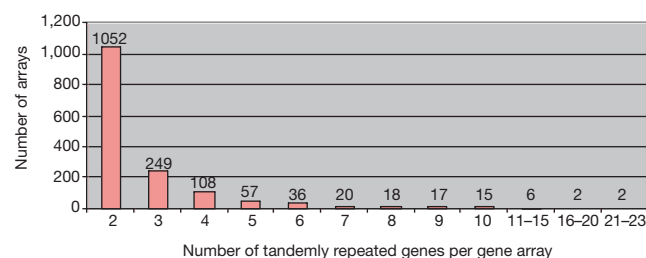


Figure 3 Distribution of tandemly repeated gene arrays in the *Arabidopsis* genome. Tandemly repeated gene arrays were identified using the BLASTP program with a threshold of $E < 10^{-20}$. One unrelated gene among cluster members was tolerated. The histogram gives the number of clusters in the genome containing 2 to n similar gene units in tandem.

found in the DNA-based alignment (Fig. 4), and these data extend earlier findings^{4,5,31}. The extent of sequence conservation of the duplicated genes varies greatly, with 6,303 (37%) of the 17,193 genes in the segments classified as highly conserved ($E < 10^{-30}$) and a further 1,705 (10%) showing less significant similarity up to $E < 10^{-5}$. The proportion of homologous genes in each duplicated segment also varies widely, between 20% and 47% for the highly conserved class of genes. In many cases, the number of copies of a gene and its counterpart differ (for example, one copy on one chromosome and multiple copies on the other; see Supplementary Information Fig. 2); this could be due to either tandem duplication or gene loss after the segmental duplication.

What does the duplication in the *Arabidopsis* genome tell us about the ancestry of the species? Polyploidy occurs widely in plants and is proposed to be a key factor in plant evolution³². As the majority of the *Arabidopsis* genome is represented in duplicated (but not triplicated) segments, it appears most likely that *Arabidopsis*, like maize, had a tetraploid ancestor³³. A comparative sequence analysis of *Arabidopsis* and tomato estimated that a duplication occurred ~112 Myr ago to form a tetraploid³⁴. The degrees of conservation of the duplicated segments might be due to divergence from an ancestral autotetraploid form, or might reflect differences present in an allotetraploid ancestor. It is also possible, however, that several independent segmental duplication events took place instead of tetraploid formation and stabilization.

The diploid genetics of *Arabidopsis* and the extensive divergence of the duplicated segments have masked its evolutionary history. The determination of *Arabidopsis* gene functions must therefore be pursued with the potential for functional redundancy taken into account. The long period of time over which genome stabilization has occurred has, however, provided ample opportunity for the divergence of the functions of genes that arose from duplications.

Comparative analysis of *Arabidopsis* accessions

Comparing the multiple accessions of *Arabidopsis* allows us to identify commonly occurring changes in genome microstructure. It also enables the development of new molecular markers for genetic mapping. High rates of polymorphism between *Arabidopsis* accessions, including both DNA sequence and copy number of tandem arrays, are prevalent at loci involved in disease resistance³⁵. This has been observed for other plant species, and such loci are thought to serve as templates for illegitimate recombination

to create new pathogen response specificities³⁶. We carried out a comparative analysis between 82 Mb of the genome sequence of *Arabidopsis* accession Columbia (Col-0) and 92.1 Mb of non-redundant low-pass (twofold redundant) sequence data of the genomic DNA of accession Landsberg *erecta* (Ler). We identified two classes of differences between the sequences: single nucleotide polymorphisms (SNPs), and insertion-deletions (InDels). As we used high stringency criteria, our results represent a minimum estimate of numbers of polymorphisms between the two genomes.

In total, we detected 25,274 SNPs, representing an average density of 1 SNP per 3.3 kb. Transitions (A/T–G/C) represented 52.1% of the SNPs, and transversions accounted for the remainder: 17.3% for A/T–T/A, 22.7% for A/T–C/G and 7.9% for C/G–G/C. In total, we detected 14,570 InDels at an average spacing of 6.1 kb. They ranged from 2 bp to over 38 kilobase-pairs, although 95% were smaller than 50 bp. Only 10% of the InDels were co-located with simple sequence repeats identified with the program Sputnik. An analysis of 416 relative insertions greater than 250 bp in Col-0 showed that 30% matched transposon-related proteins, indicating that a substantial proportion of the large InDels are the result of transposon insertion or excision. Many InDels contained entire active genes not related to transposons. Half of such genes absent from corresponding positions in the Col-0 sequence were found elsewhere on the genome of *Ler*. This indicates that genes have been transferred to new genomic locations.

Gene structures are often affected by small InDels and SNPs. The positions of SNPs and InDels were mapped relative to 87,427 exons and 70,379 introns annotated in the Col-0 sequence. SNPs were found in exons, introns and intergenic regions at frequencies of 1 SNP per 3.1, 2.2 and 3.5 kb, respectively. The frequencies for InDels were 1 per 9.3, 3.1 and 4.3 kb, respectively. Polymorphisms were detected in 7% of exons, and alter the spliced sequences of 25% of the predicted genes. For InDels in exons, insertion lengths divisible by three are prevalent for small insertions (< 50 bp), indicating that many proteins can withstand small insertions or deletions of amino acids without loss of function.

Our analyses show that sequence polymorphisms between accessions of *Arabidopsis* are common, and that they occur in both coding and non-coding regions. We found evidence for the relocation of genes in the genome, and for changes in the complement of transposable elements. The data presented here are available at <http://www.arabidopsis.org/cereon/>.

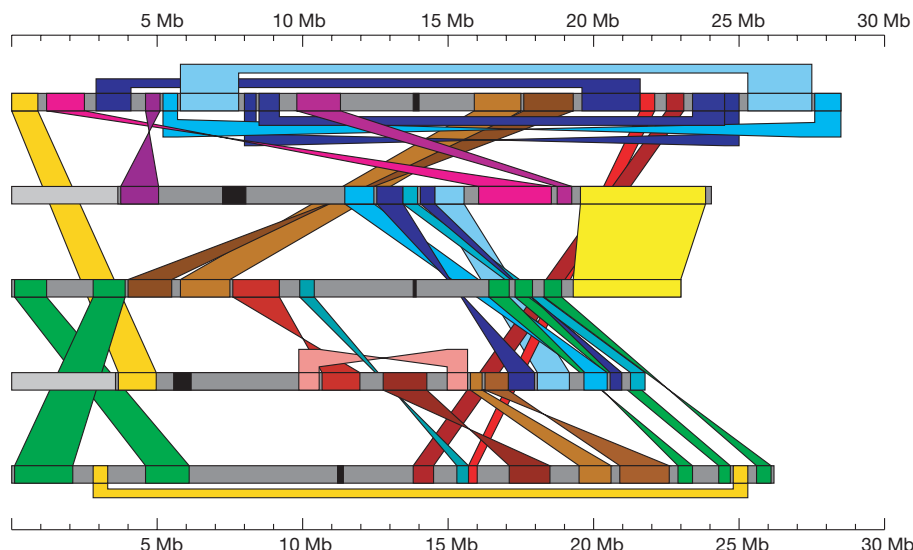


Figure 4 Segmentally duplicated regions in the *Arabidopsis* genome. Individual chromosomes are depicted as horizontal grey bars (with chromosome 1 at the top), centromeres are marked black. Coloured bands connect corresponding duplicated

segments. Similarity between the rDNA repeats are excluded. Duplicated segments in reversed orientation are connected with twisted coloured bands. The scale is in megabases.

Comparison of *Arabidopsis* and other plant genera

Comparative genetic mapping can reveal extensive conservation of genome organization between closely related species^{37,38}. The comparative analysis of plant genome microstructure reveals much about the evolution of plant genomes and provides unprecedented opportunities for crop improvement by establishing the detailed structures of, and relationships between, the genomes of crops and *Arabidopsis*.

The lineages leading to *Arabidopsis* and *Capsella rubella* (shepherd's purse) diverged between 6.2 and 9.8 Myr ago, and the gene content and genome organization of *C. rubella* is very similar to that of *Arabidopsis*³⁹, including the large-scale duplications. Alignment of *Arabidopsis* complementary DNA and EST sequences with genomic DNA sequences of *Arabidopsis* and *C. rubella* showed conservation of exon length and intron positions. Coding sequences predicted from these alignments differed from the annotated *Arabidopsis* gene sequences in two out of five cases.

The ancestral lineages of *Arabidopsis* and the *Brassica* (cabbage and mustard) genera diverged 12.2–19.2 Myr ago⁴⁰. *Brassica* genes show a high level of nucleotide conservation with their *Arabidopsis* orthologues, typically more than 85% in coding regions⁴⁰. The structure of *Brassica* genomes resembles that of *Arabidopsis*, but with extensive triplication and rearrangement⁴¹, and extensive divergence of microstructure (Supplementary Information Fig. 3). The divergence between the genomes of *Arabidopsis* and *Brassica oleracea* is in striking contrast to that observed between *Arabidopsis* and *C. rubella*, although the time since divergence is only twofold greater. This accelerated rate of change in triplicated segments of the genome of *B. oleracea* indicates that polyploidy fosters rapid chromosomal evolution.

The *Arabidopsis* and tomato lineages diverged roughly 150 Myr ago, and comparative sequence analysis of segments of their genomes has revealed complex relationships³⁴. Four regions of the *Arabidopsis* genome are related to each other and to one region in the tomato genome, suggesting that two rounds of duplication may

have occurred in the *Arabidopsis* lineage. The extensive duplication described here supports the proposal that the more recent of these duplications, estimated to have occurred ~112 Myr ago, was the result of a polyploidization event. The lineages of *Arabidopsis* and rice diverged ~200 Myr ago⁴². Three regions of the genome of *Arabidopsis* were related to each other and to one region in the rice genome, providing further evidence for multiple duplication events^{43,44}.

The frequent occurrence of tandem gene duplications and the apparent deletion of single genes, or small groups of adjacent genes, from duplicated regions suggests that unequal crossing over may be a key mechanism affecting the evolution of plant genome microstructure. However, the segmental inversions and gene translocations in the genomes of both rice and *B. oleracea* that are not found in *Arabidopsis* indicate that additional mechanisms may be involved⁴⁰.

Integration of the three genomes in the plant cell

The three genomes in the plant cell—those of the nucleus, the plastids (chloroplasts) and the mitochondria—differ markedly in gene number, organization and stability. Plastid genes are densely packed in an order highly conserved in all plants⁴⁵, whereas mitochondrial genes⁴⁶ are widely dispersed and subjected to extensive recombination.

Organelle genomes are remnants of independent organisms—plastids are derived from the cyanobacterial lineage and mitochondria from the α -Proteobacteria. The remaining genes in plastids include those that encode subunits of the photosystem and the electron transport chain, whereas the genes in mitochondria encode essential subunits of the respiratory chain. Both organelles contain sets of specific membrane proteins that, together with housekeeping proteins, account for 61% of the genes in the chloroplast and 88 % in the mitochondrion (Table 4). The balances are involved in transcription and translation.

The number of proteins encoded in the nucleus likely to be found

Table 3 *Arabidopsis* genes with similarities to human disease genes

Human disease gene	E value	Gene code	<i>Arabidopsis</i> hit
Darier–White, SERCA	5.9×10^{-272}	T2711_16	Putative calcium ATPase
Xeroderma Pigmentosum, D-XPD	7.2×10^{-228}	F15K9_19	Putative DNA repair protein
Xeroderma pigment, B-ERCC3	9.6×10^{-214}	AT5g41360	DNA excision repair cross-complementing protein
Hyperinsulinism, ABCC8	7.1×10^{-188}	F20D22_11	Multidrug resistance protein
Renal tubul. acidosis, ATP6B1	1.0×10^{-182}	AT4g38510	Probable H ⁺ -transporting ATPase
HDL deficiency 1, ABCA1	2.4×10^{-181}	AT2g41700	Putative ABC transporter
Wilson, ATP7B	7.6×10^{-181}	AT5g44790	ATP-dependent copper transporter
Immunodeficiency, DNA Ligase 1	8.2×10^{-172}	T6D22_10	DNA ligase
Stargardt's, ABCA4	2.8×10^{-168}	At2g41700	Putative ABC transporter
Ataxia telangiectasia, ATM	3.1×10^{-168}	AT3g48190	Ataxia telangiectasia mutated protein AtATM
Niemann–Pick, NPC1	1.2×10^{-166}	F7F22_1	Niemann–Pick C disease protein-like protein
Menkes, ATP7A	1.1×10^{-153}	F2K11_17	ATP-dependent copper transporter, putative
HNPCC*, MLH1	1.5×10^{-150}	AT4g09140	MLH1 protein
Deafness, hereditary, MYO15	2.7×10^{-150}	At2g31900	Putative unconventional myosin
Fam, cardiac myopathy, MYH7	6.5×10^{-147}	T1G11_14	Putative myosin heavy chain
Xeroderma Pigmentosum, F-XPF	1.4×10^{-146}	AT5g41150	Repair endonuclease (gb)AAFO1274.1)
G6PD deficiency, G6PD	7.6×10^{-137}	AT4g00760	Glucose-6-phosphate dehydrogenase
Cystic fibrosis, ABCC7	2.3×10^{-135}	AT3g62700	ABC transporter-like protein
Glycerol kinase defic, GK	7.9×10^{-135}	T21F11_21	Putative glycerol kinase
HNPCC, MSH3	6.6×10^{-134}	AT4g25540	Putative DNA mismatch repair protein
HNPCC, PMS2	5.1×10^{-128}	AT4g02460	No title
Zellweger, PEX1	4.1×10^{-125}	AT5g08470	Putative protein
HNPCC, MSH6	9.6×10^{-122}	AT4g02070	G/T DNA mismatch repair enzyme
Bloom, BLM	4.4×10^{-109}	T19D16_15	DNA helicase isolog
Finnish amyloidosis, GSN	2.2×10^{-107}	AT5g57320	Villin
Chediak–Higashi, CHS1	5.8×10^{-99}	F10O3_11	Putative transport protein
Xeroderma Pigmentosum, G-XPG	7.1×10^{-89}	AT3g28030	Hypothetical protein
Bare lymphocyte, ABCB3	1.3×10^{-84}	AT5g39040	ABC transporter-like protein
Citrullinemia, type I, ASS	3.2×10^{-83}	AT4g24830	Argininosuccinate synthase-like protein
Coffin–Lowry, RPS6KA3	5.2×10^{-81}	AT3g08720	Putative ribosomal-protein S6 kinase (ATPK19)
Keratoderma, KRT9	8.5×10^{-81}	AT3g17050	Unknown protein
Myotonic dystrophy, DM1	1.4×10^{-76}	At2g20470	Putative protein kinase
Bartter's, SLC12A1	1.6×10^{-75}	F26G16_9	Cation-chloride co-transporter, putative
Dents, CLCN5	3.3×10^{-74}	AT5g26240	CLC-d chloride channel protein
Diaphanous 1, DAPH1	1.9×10^{-73}	68069_m00158	Hypothetical protein
AKT2	6.9×10^{-72}	AT3g08730	Putative ribosomal-protein S6 kinase (ATPK6)

in organelles was predicted using default settings on TargetP (Table 1). Many nuclear gene products that are targeted to either (or both) organelles were originally encoded in the organelle genomes and were transferred to the nuclear genome during evolutionary history. A large number also appear to be of eukaryotic origin, with functions such as protein import components, which were probably not required by the free-living ancestors of the endosymbionts.

To identify nuclear genes of possible organellar ancestry, we compared all predicted *Arabidopsis* proteins to all proteins from completed genomes including those from plastids and mitochondria (Supplementary Information Table 2). This search identified proteins encoded by the *Arabidopsis* nuclear genome that are most similar to proteins encoded by other species' organelle genomes (14 mitochondrial and 44 plastid). These represent organelle-to-nuclear gene transfers that have occurred sometime after the divergence of the organelle-containing lineages⁴⁷. There is a great excess of nuclear encoded proteins most similar to proteins from the cyanobacteria *Synechocystis* (Supplementary Information Fig. 4; 806 *Arabidopsis* predicted proteins matching 404 different *Synechocystis* proteins, providing further evidence of a genome duplication). These 806 *Arabidopsis* predicted proteins, and many others of greatly diverse function, are possibly of plastid descent. Through searches against proteins from other cyanobacteria (with incompletely sequenced genomes), we identified 69 additional genes of possibly plastid descent. Only 25% of these putatively plastid-derived proteins displayed a target peptide predicted by TargetP, indicating potential cytoplasmic functions for most of these genes.

The difference between predicted plastid-targeted and predicted plastid-derived genes indicates that there is a probable overestimation by *ab initio* targeting prediction methods and a lack of resolution with respect to destination organelles, the possible extensive divergence of some endosymbiont-derived genes in the nuclear genome, the co-opting of nuclear genes for targeting to organelles, and cytoplasmic functions for cyanobacteria-derived proteins. Clearly more refined tools and extensive experimentation is required to catalogue plastid proteins.

The transfer of genes between genomes still continues (Supplementary Information Table 3). Plastid DNA insertions in the nucleus (17 insertions totalling 11 kb) contain full-length genes encoding proteins or tRNAs, fragments of genes and an intron as well as intergenic regions. Subsequent reshuffling in the nucleus is illustrated by the *atpH* gene, which was originally transferred completely, but is now in two pieces separated by 2 kb. The 13 small mitochondrial DNA insertions total 7 kb in addition to the large insertion close to the centromere of chromosome 2 (ref. 3). The high level of recombination in the mitochondrial genome may account for these events.

Transposable elements

Transposons, which were originally identified in maize by Barbara McClintock, have been found in all eukaryotes and prokaryotes. A

subset of transposons replicate through an RNA intermediate (class I), whereas others move directly through a DNA form (class II). Transposons are further classified by similarity either between their mobility genes or between their terminal and/or internal motifs, as well as by the size and sequence of their target site. Internally deleted elements can often be mobilized in *trans* by fully functional elements.

Transposons in *Arabidopsis* account for at least 10% of the genome, or about one-fifth of the intergenic DNA. The *Arabidopsis* genome has a wealth of class I (2,109) and II (2,203) elements, including several new groups (1,209 elements; Supplementary Information Table 4). Mobile histories for many elements were obtained by identifying regions of the genome with significant similarity to 'empty' target sites (RESites) thus providing high-resolution information concerning the termini and target site duplications^{48,49}. These regions were readily detected because of the propensity of transposons to integrate into repeats and because of duplications in the genome sequence. In several cases, genes appear to have been included as 'passengers' in transposable units⁴⁸. In some cases, shared sequence similarity, coding capacity and RESites attest to recent activity of transposable elements in the *Arabidopsis* genome. Only about 4% of the complete elements identified correspond to an EST, however, suggesting that most are not transcribed.

Transposable elements found in many other plant genomes are well represented in *Arabidopsis*, including *copia*- and *gypsy*-like long terminal repeat (LTR) retrotransposons, long interspersal nuclear elements (LINEs); short interspersed nuclear elements (SINEs), *hobo/Activator/Tam3* (*hAT*)-like elements, CACTA-like elements and miniature inverted-repeat transposable elements (MITES). Although usually small in size, some larger *Tourist*-like MITES contain open reading frames (ORFs) with similarity to the transposases of bacterial insertion sequences⁴⁸. *Basho* and many *Mutator*-like elements (MULEs), first discovered in the *Arabidopsis* sequence, represent structurally unique transposons⁴⁸⁻⁵⁰. *Basho* elements have a target site preference for mononucleotide 'A' and wide distribution among plants^{48,51}. MULEs exhibit a high level of sequence diversity and members of most groups lack long terminal inverted repeats (TIRs). Phylogenetic analysis of the *Arabidopsis* MURA-like transposases suggests that TIR-containing MULEs are more closely related to one another than to MULEs lacking TIRs^{49,52}.

For many plants with large genomes, class I retrotransposons contribute most of the nucleotide content⁵³. In the small *Arabidopsis* genome, class I elements are less abundant and primarily occupy the centromere. In contrast, *Basho* elements and class II transposons such as MITES and MULEs predominate on the periphery of pericentromeric domains (Fig. 5). In class II transposons, MULEs and CACTA elements are clustered near centromeres and heterochromatic knobs, whereas MITES and *hAT* elements have a less pronounced bias. The distribution pattern of transposable elements observed in *Arabidopsis* may reflect different types of pericentromeric heterochromatin regions and may be similar to those found in animals.

Numerous centromeric satellite repeats are located between each chromosome arm and have not yet been sequenced, but are represented in part by unanchored BAC contigs (R. Martienssen and M. Marra, unpublished data). End sequence suggests that these domains contain many more class I than class II elements, consistent with the distribution reported here (K. Lemcke and R. Martienssen, unpublished data). We do not know the significance of the apparent paucity of elements in telomeric regions and in the region flanking the rDNA repeats on chromosome 4 (but not on chromosome 2).

Overall, transposon-rich regions are relatively gene-poor and have lower rates of recombination and EST matches, indicating a correlation between low gene expression, high transposon density and low recombination⁵¹. The role of transposons in genome

Table 4 General features of genes encoded by the three genomes in *Arabidopsis*

	Nucleus/cytoplasm	Plastid	Mitochondria
Genome size	125 Mb	154 kb	367 kb
Genome equivalent/cell	2	560	26
Duplication	60%	17%	10%
Number of protein genes	25,498	79	58
Gene order	Variable, but syntenic	Conserved	Variable
Density (kb per protein gene)	4.5	1.2	6.25
Average coding length	1,900 nt	900 nt	860 nt
Genes with introns	79%	18.4%	12%
Genes/pseudogenes	1/0.03	1/0	1/0.2–0.5
Transposons (% of total genome size)	14%	0%	4%

organization and chromosome structure can now be addressed in a model organism known to undergo DNA methylation and other forms of chromatin modification thought to regulate transposition⁵².

rDNA, telomeres and centromeres

Nucleolar organizers (NORs) contain arrays of unit repeats encoding the 18S, 5.8S and 25S ribosomal RNA genes and are transcribed by RNA polymerase I. Together with 5S RNA, which is transcribed by RNA polymerase III, these rRNAs form the structural and catalytic cores of cytoplasmic ribosomes. In *Arabidopsis*, the NORs juxtapose the telomeres of chromosomes 2 and 4, and comprise uninterrupted 18S, 5.8S and 25S units all orientated on the chromosomes in the same direction⁵⁴. In contrast, the 5S rRNA genes are localized to heterogeneous arrays in the centromeric regions of chromosomes 3, 4 and 5 (ref. 55; and Fig. 6). Both NORs are roughly 3.5–4.0 megabase-pairs and comprise ~350–400 highly methylated rRNA gene units, each ~10 kb (ref. 54). The sequence between the euchromatic arms and NORs has been determined. Elsewhere in the genome, only one other 18S, 5.8S, 25S rRNA gene unit was identified in centromere 3. Although minor variations in sequence length and composition occur in the NOR repeats, these variants are highly clustered, supporting a model of sequence maintenance through concerted evolution⁵⁵.

Arabidopsis telomeres are composed of CCCTAA repeats and average ~2–3 kb (ref. 56). For TEL4N (telomere 4 North), consensus repeats are adjacent to the NOR; the remaining telomeres are typically separated from coding sequences by repetitive subtelomeric regions measuring less than 4 kb. Imperfect telomere-like arrays of up to 24 kb are found elsewhere in the genome, particularly

near centromeres. These arrays might affect the expression of nearby genes and may have resulted from ancient rearrangements, such as inversions of the chromosome arms.

Centromere DNA mediates chromosome attachment to the meiotic and mitotic spindles and often forms dense heterochromatin. Genetic mapping of the regions that confer centromere function provided the markers necessary to precisely place BAC clones at individual centromeres¹⁷; 69 clones were targeted for sequencing, resulting in over 5 Mb of DNA sequence from the centromeric regions. The unsequenced regions of centromeres are composed primarily of long, homogeneous arrays that were characterized previously with physical⁵⁷ and genetic mapping¹⁷ and contain over 3 Mb of repetitive arrays, including the 180-bp repeats and 5S rDNA⁵¹ (Fig. 6).

Arabidopsis centromeres, like those of many higher eukaryotes, contain numerous repetitive elements including retroelements, transposons, microsatellites and middle repetitive DNA¹⁷. These repeats are rare in the euchromatic arms and often most abundant in pericentromeric DNA. The repeats, affinity for DNA-binding dyes, dense methylation patterns and inhibition of homologous recombination indicate that the centromeric regions are highly heterochromatic, and such regions are generally viewed as very poor environments for gene expression. Unexpectedly, we found at least 47 expressed genes encoded in the genetically defined centromeres of *Arabidopsis* (<http://preuss.bsd.uchicago.edu/arabidopsis.genome.html>). In several cases, these genes reside on islands of unique sequence flanked by repetitive arrays, such as 180-bp or 5S rDNA repeats. Among the genes encoded in the centromeres are members of 11 of the 16 functional categories that comprise the proteome. The centromeres are not subject to recombination; consequently, genes residing in these regions probably exhibit unique patterns of molecular evolution.

The function of higher eukaryotic centromeres may be specified by proteins that bind to centromere DNA, by epigenetic modifications, or by secondary or higher order structures. A pairwise comparison of the non-repetitive portions of all five centromeres showed they share limited (1–7%) sequence similarity. Forty-one families of small, conserved centromere sequences (AtCCS, see <http://preuss.bsd.uchicago.edu/arabidopsis.genome.html>) are enriched in the centromeric and pericentromeric regions and differ from sequences found in the centromeres of other eukaryotes. Molecular and genetic assays will be required to determine whether these conserved motifs nucleate *Arabidopsis* centromere activity. Apart from the AtCCS sequences, most centromere DNA is not shared between chromosomes, complicating efforts to derive clear evolutionary relationships. In contrast, genetic and cytological assays indicate that homologous centromeres are highly conserved among *Arabidopsis* accessions, albeit subject to rearrangements such as inversions to form knobs^{5,58,59} and insertions⁴. Further investigation of centromere DNA promises to yield information on the evolutionary forces that act in regions of limited recombination, as well as an improved understanding of the role of DNA sequence patterns in chromosome segregation.

Membrane transport

Transporters in the plasma and intracellular membranes of *Arabidopsis* are responsible for the acquisition, redistribution and compartmentalization of organic nutrients and inorganic ions, as well as for the efflux of toxic compounds and metabolic end products, energy and signal transduction, and turgor generation. Previous genomic analyses of membrane transport systems in *S. cerevisiae* and *C. elegans* led to the identification of over 100 distinct families of membrane transporters^{60,61}. We compared membrane transport processes between *Arabidopsis*, animals, fungi and prokaryotes, and identified over 600 predicted membrane transport systems in *Arabidopsis* (<http://www-biology.ucsd.edu/~ipaulsen/transport/>), a similar number to that of *C. elegans*

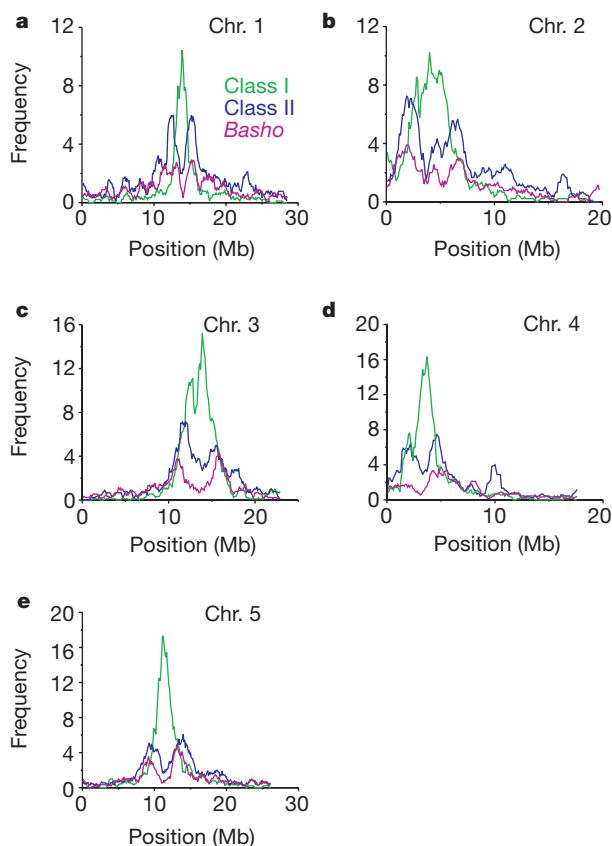


Figure 5 Distribution of class I, II and *Bashed* transposons in *Arabidopsis* chromosomes. The frequency of class I retroelements (green), class II DNA transposons (blue) and *Bashed* elements (purple) are shown at 100-kb intervals along the five chromosomes (a–e) of *Arabidopsis*.

(~700 transporters) and over twofold greater than either *S. cerevisiae* or *E. coli* (~300 transporters).

We compared the transporter complement of *Arabidopsis*, *C. elegans* and *S. cerevisiae* in terms of energy coupling mechanisms (Fig. 7a). Unlike animals, which use a sodium ion P-type ATPase pump to generate an electrochemical gradient across the plasma membrane, plants and fungi use a proton P-type ATPase pump to form a large membrane potential (~250 mV)⁶². Consequently, plant secondary transporters are typically coupled to protons rather than to sodium⁶³. Compared with *C. elegans*, *Arabidopsis* has a surprisingly high percentage of primary ATP-dependent transporters (12% and 21% of transporters, respectively), reflecting increased numbers of P-type ATPases involved in metal ion transport and ABC ATPases proposed to be involved in sequestering unusual metabolites and drugs in the vacuole or in other intracellular compartments. These processes may be necessary for pathogen defence and nutrient storage.

About 15% of the transporters in *Arabidopsis* are channel proteins, five times more than in any single-celled organism but half the number in *C. elegans* (Fig. 7b). Almost half of the *Arabidopsis* channel proteins are aquaporins, and *Arabidopsis* has 10-fold more Mfamily major intrinsic protein (MIP) family water channels than any other sequenced organism. This abundance emphasizes the importance of hydraulics in a wide range of plant processes, including sugar and nutrient transport into and out of the vasculature, opening of stomatal apertures, cell elongation and epinastic movements of leaves and stems. Although *Arabidopsis* has a diverse range of metal cation transporters, *C. elegans* has more, many of which function in cell–cell signalling and nerve signal transduction. *Arabidopsis* also possesses transporters for inorganic anions such as phosphate, sulphate, nitrate and chloride, as well as for metal cation channels that serve in signal transduction or cell homeostasis. Compared with other sequenced organisms, *Arabidopsis* has 10-fold more predicted peptide transporters, primarily of the proton-dependent oligopeptide transport (POT) family, emphasizing the importance of peptide transport or indicating that there is broader substrate specificity than previously realized. There are nearly 1,000 *Arabidopsis* genes encoding Ser/Thr protein kinases, suggesting that peptides may have an important role in plant signalling⁶⁴.

Virtually no transporters for carboxylates, such as lactate and pyruvate, were identified in the *Arabidopsis* genome. About 12% of the transporters were predicted to be sugar transporters, mostly consisting of paralogues of the MFS family of hexose transporters. Notably, *S. cerevisiae*, *C. elegans* and most prokaryotes use APC family transporters as their principle means of amino-acid

transport, but *Arabidopsis* appears to rely primarily on the AAAP family of amino-acid and auxin transporters. More than 10% of the transporters in *Arabidopsis* are homologous to drug efflux pumps; these probably represent transporters involved in the sequestration into vacuoles of xenobiotics, secondary metabolites, and breakdown products of chlorophyll.

Surprisingly, *Arabidopsis* has close homologues of the human ABC TAP transporters of antigenic peptides for presentation to the major histocompatibility complex (MHC). In *Arabidopsis*, these transporters may be involved in peptide efflux, or more speculatively, in some form of cell-recognition response. *Arabidopsis* also has 10-fold more members of the multi-drug and toxin extrusion (MATE) family than any other sequenced organism; in bacteria, these transporters function as drug efflux pumps. Curiously, *Arabidopsis* has several homologues of the *Drosophila* RND transporter family Patched protein, which functions in segment polarity, and more than ten homologues of the *Drosophila* ABC family eye pigment transporters. In plants, these are presumably involved in intracellular sequestration of secondary metabolites.

DNA repair and recombination

DNA repair and recombination pathways have many functions in different species such as maintaining genomic integrity, regulating mutation rates, chromosome segregation and recombination, genetic exchange within and between populations, and immune system development. Comparing the *Arabidopsis* genome with other species⁶⁵ indicates that *Arabidopsis* has a similar set of DNA repair and recombination (RAR) genes to most other eukaryotes. The pathways represented include photoreactivation, DNA ligation, non-homologous end joining, base excision repair, mismatch excision repair, nucleotide excision repair and many aspects of DNA recombination (Supplementary Information Table 5). The *Arabidopsis* RAR genes include homologues of many DNA repair genes that are defective in different human diseases (for example, hereditary breast cancer and non-polyposis colon cancer, xeroderma pigmentosum and Cockayne's syndrome).

One feature that sets *Arabidopsis* apart from other eukaryotes is the presence of additional homologues of many RAR genes. This is seen for almost every major class of DNA repair, including recombination (four RecA), DNA ligation (four DNA ligase I), photoreactivation (one class II photolyase and five class I photolyase homologues) and nucleotide excision repair (six RPA1, two RPA2, two Rad25, three TFB1 and four Rad23). This is most striking for genes with probable roles in base excision repair. *Arabidopsis* encodes 16 homologues of DNA base glycosylases (enzymes that

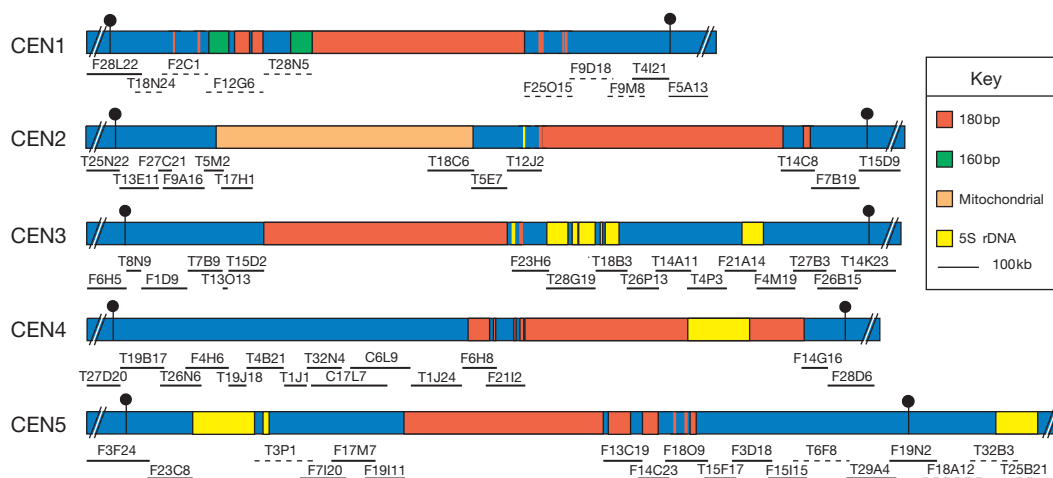


Figure 6 Predicted centromere composition. Genetically defined centromere boundaries are indicated by filled circles; fully and partially assembled BAC sequences are represented by solid and dashed black lines, respectively. Estimates of repeat sizes within

the centromeres were derived from consideration of repeat copy number, physical mapping and cytogenetic assays.

recognize abnormal DNA bases and cleave them from the sugar-phosphate backbone)—more than any other species known. This includes several homologues of each of three families of alkylation damage base glycosylases: two of the *S. cerevisiae* MPG; six of the *E. coli* TagI; and two of the *E. coli* AlkA. *Arabidopsis* also encodes three homologues of the apurinic-apyrimidinic (AP) endonuclease Xth. AP endonucleases continue the base excision repair started by glycosylases by cleaving the DNA backbone at abasic sites.

Evolutionary analysis indicates that some of the extra copies of RAR genes in *Arabidopsis* originated through relatively recent gene duplications—because many of the sets of genes are more closely related to each other than to their homologues in any other species. As duplication is frequently accompanied by functional divergence, the duplicate (paralogous) genes may have different repair specificities or may have evolved functions that are outside RAR functions (as is the case for two of the five class I photolyase homologues, which function as blue-light receptors). In most cases, it is not known whether the paralogous gene copies have different functions. The presence of multiple paralogues might also allow functional redundancy or a greater repair or recombination capacity.

The multiplicity of RAR genes in *Arabidopsis* is also partly due to the transfer of genes from the organellar genomes to the nucleus. Repair gene homologues that appear to be of chloroplast origin (Supplementary Information Tables 2 and 5) include the recombination proteins RecA, RecG and SMS, two class I photolyase homologues, Fpg, two MutS2 proteins, and the transcription-repair coupling factor Mfd. Two of these (RecA and Fpg) are involved in RAR functions in the plastid, suggesting that the others may be as well. The finding of an Mfd orthologue of cyanobacterial descent is surprising. In *E. coli*, Mfd couples nucleotide excision repair carried out by UvrABC to transcription, leading to the rapid repair of DNA damage on the transcribed strand of transcribed genes⁶⁶. The absence of orthologues of UvrABC in *Arabidopsis* renders the function of Mfd difficult to predict. The presence of Mfd but not UvrABC has been reported for only one other species, a bacterial endosymbiont of the pea aphid.

Other nuclear-encoded *Arabidopsis* DNA repair gene homologues are evolutionarily related to genes from α -Proteobacteria, and thus may be of mitochondrial descent. In particular, the six homologues of the alkyl-base glycosylase TagI appear to be the result of a large expansion in plants after transfer from the mitochondrial genome. Whether any of these TagI homologues function in the repair and maintenance of mitochondrial DNA has not been determined. More detailed phylogenetic analysis may reveal additional *Arabidopsis* RAR genes to be of organellar ancestry.

There are some notable absences of proteins important for RAR in other species, including alkyltransferases, MSH4, RPA3 and many components of TFIIH (TFB2, TFB3, TFB4, CCL1, Kin28). Nevertheless, *Arabidopsis* shows many similarities to the set of DNA repair genes found in other eukaryotes, and therefore offers an experimental system for determining the functions of many of these proteins, in part through characterization of mutants defective in DNA repair⁶⁷.

Gene regulation

Eukaryotic gene expression involves many nuclear proteins that modulate chromatin structure, contribute to the basal transcription machinery, or mediate gene regulation in response to developmental, environmental or metabolic cues. As predicted by sequence similarity, more than 3,000 such proteins may be encoded by the *Arabidopsis* genome, suggesting that it has a comparable complexity of gene regulation to other eukaryotes. *Arabidopsis* has an additional level of gene regulation, however, with DNA methylation potentially mediating gene silencing and parental imprinting.

Plants have evolved several variations on chromatin remodelling proteins, such as the family of HD2 histone deacetylases⁶⁸. Although *Arabidopsis* possesses the usual number of SNF2-type chromatin

remodelling ATPases, which regulate the expression of nearly all genes, there are significant structural differences between yeast and metazoan SNF2-type genes and their orthologues in *Arabidopsis*. DDM1, a member of the SNF2 superfamily, and MOM1, a gene with similarity to the SNF2 family, are involved in transcriptional gene silencing in *Arabidopsis*. MOM1 has no clear orthologue in fungal or metazoan genomes.

Consistent with its methylated DNA, *Arabidopsis* possesses eight DNA methyltransferases (DMTs). Two of the three types are orthologous to mammalian DMT⁶⁹ whereas one, chromomethyltransferase⁷⁰, is unique to plants. No DMTs are found in yeast or *C. elegans*, although two DMT-like genes are found in *Drosophila*⁷¹. *Arabidopsis* also encodes eight proteins with methyl-DNA-binding domains (MBDs). Despite lacking methylated DNA, *Drosophila* encodes four MBD proteins and *C. elegans* has two. These differences in chromatin components are likely to reflect important differences in chromatin-based regulatory control of gene expression in eukaryotes (Supplementary Information Table 6; <http://Ag.Arizona.Edu/chromatin/chromatin.html>).

The *Arabidopsis* genome encodes transcription machinery for the three nuclear DNA-dependent RNA polymerase systems typical of eukaryotes (Supplementary Information Table 6). Transcription by RNA polymerases II and III appears to involve the same machinery as is used in other eukaryotes; however, most transcription factors for RNA polymerase I are not readily identified. Only two polymerase I regulators (other than polymerase subunits and TATA-binding protein) are apparent in *Arabidopsis*, namely homologues of yeast RRN3 and mouse TTF-1. All eukaryotes examined to date have distinct genes for the largest and second largest subunits of polymerase I, II and III. Unexpectedly, *Arabidopsis* has two genes encoding a fourth class of largest subunit and second-largest subunit (Supplementary Information Fig. 5). It will be interesting to determine whether the atypical subunits comprise a polymerase that has a plant-specific function. Four genes encoding single-subunit plastid or mitochondrial RNA polymerases have been identified in *Arabidopsis* (Supplementary Information Table 6). Genes for the bacterial β -, β' - and α -subunits of RNA polymerase are also present, as are homologues of various σ -factors, and these proteins may regulate chloroplast gene expression. Mutations in the *Sde-1* gene, encoding RNA-dependent RNA polymerase (RdRp), lead to defective post-transcriptional gene silencing⁷². We also identified five more closely related RdRp genes.

Our analysis, using both similarity searches and domain matches, has identified 1,709 proteins with significant similarity to known classes of plant transcription factors classified by conserved DNA-binding domains. This analysis used a consistent conservative threshold that probably underestimates the size of families of diverse sequence. This class of protein is the least conserved among all classes of known proteins, showing only 8–23% similarity to transcription factors in other eukaryotes (Fig. 2b). This reduced similarity is due to the absence of certain classes of transcription factors in *Arabidopsis* and large numbers of plant-specific transcription factors. We did not detect any members of several widespread families of transcription factors, such as the REL (Rel-like DNA-binding domain) homology region proteins, nuclear steroid receptors and forkhead-winged helix and POU (Pit-1, Oct- and Unc-8b) domain families of developmental regulators. Conversely, of 29 classes of *Arabidopsis* transcription factors, 16 appear to be unique to plants (Supplementary Information Table 6). Several of these, such as the AP2/EREBP-RAV, NAC and ARF-AUX/IAA families, contain unique DNA-binding domains, whereas others contain plant-specific variants of more widespread domains, such as the DOF and WRKY zinc-finger families and the two-repeat MYB family.

Functional redundancy among members of large families of closely related transcription factors in *Arabidopsis* is a significant potential barrier to their characterization⁷³. For example, in the

SHATTERPROOF and SEPALLATA families of MADS box transcription factors, all genes must be defective to produce visible mutant phenotypes^{74,75}. These functionally redundant genes are found on the segmental duplications described above. Our analyses, together with the significant sequence similarity found in large families of transcription factors such as the R2R3-repeat MYB and WRKY families, suggest that strategies involving overexpression will be important in determining the functions of members of transcription factor families.

Arabidopsis has two or over three times more transcription factors than identified in *Drosophila*²⁹ or *C.elegans*¹, respectively. The significantly greater extent of segmental chromosomal and local tandem duplications in the *Arabidopsis* genome generates larger gene families, including transcription factors. The partly overlapping functions defined for a few transcription factors are also likely to be much more widespread, implicating many sequence-related transcription factors in the same cellular processes. Finally, the expanded number of genes involved in metabolism, defence and environmental interaction in *Arabidopsis* (Fig. 2a), which have few counterparts in *Drosophila* and *C. elegans*, all require additional numbers and classes of transcription factors to integrate gene function in response to a vast range of developmental and environmental cues.

Cellular organization

Plant cells differ from animal cells in many features such as plastids, vacuoles, Golgi organization, cytoskeletal arrays, plasmodesmata linking cytoplasms of neighbouring cells, and a rigid polysaccharide-rich extracellular matrix—the cell wall. Because the cell wall maintains the position of a cell relative to its neighbours, both changes in cell shape and organized cell divisions, involving cytoskeleton reorganization and membrane vesicle targeting, have major roles in plant development. Plant cytokinesis is also unique in that the partitioning membrane is formed *de novo* by vesicle fusion. We compared the *Arabidopsis* genome with those of *C. elegans*,

Drosophila and yeast to glimpse the genetic basis of plant-cell-specific features.

The principal components of the plant cytoskeleton are microtubules (MTs) and actin filaments (AFs); intermediate filaments (IFs) have not been described in plants. *Arabidopsis* appears to lack genes for cyokeratin or vimentin, the main components of animal IFs, but has several variants of actin, α - and β -tubulin. The *Arabidopsis* genome also encodes homologues of chaperones that mediate the folding of tubulin and actin polypeptides in yeast and animal cells, such as the prefoldin and cytosolic chaperonin complexes and tubulin-folding cofactors. The dynamic stability of MTs and AFs is influenced by MT-associated proteins and actin-binding proteins, respectively, several of which are encoded by *Arabidopsis* genes. These include the MT-severing ATPase katanin, AF-cross-linking/bundling proteins, such as fimbrins and villins, and AF-disassembling proteins, such as profilin and actin-depolymerizing factor/cofilin. The *Arabidopsis* proteome appears to lack homologues of proteins that, in animal cells, link the actin cytoskeleton across the plasma membrane to the extracellular matrix, such as integrin, talin, spectrin, α -actinin, vitronectin or vinculin. This apparent lack of ‘anchorage’ proteins is consistent with the different composition of the cell wall and with a prominence of cortical MTs at the expense of cortical AFs in plant cells.

Plant-specific cytoskeletal arrays include interphase cortical MTs mediating cell shape, the preprophase band marking the cortical site of cell division, and the phragmoplast assisting in cytokinesis⁷⁶. Although plant cells lack structural counterparts of the yeast spindle pole body and the animal centrosome, *Arabidopsis* has homologues of core components of the MT-nucleating γ -tubulin ring complex, such as γ -tubulin, Spc97/hGCP2 and Spc98/hGCP3. *Arabidopsis* has numerous motor molecules, both kinesins and dyneins with associated dynactin complex proteins, which are presumably involved in the dynamic organization of MTs and in transporting cargo along MT tracks. There are also myosin motors that may be involved in AF-supported organelle trafficking. Essential features of the eukaryotic cytoskeleton appear to be conserved in *Arabidopsis*.

The *Arabidopsis* genome encodes homologues of proteins involved in vesicle budding, including several ARFs and ARF-related small G-proteins, large but not small ARF GEFs (adenosine ribosylation factor on guanine nucleotide exchange factor), adapter proteins, and coat proteins of the COP and non-COP types. *Arabidopsis* also has homologues of proteins involved in vesicle docking and fusion, including SNAP receptors (SNAREs), N-ethylmaleimide-sensitive factor (NSF) and Cdc48-related ATPases, accessory proteins such as Sec1 and soluble NSF attachment protein (SNAP), and Rab-type GTPases. The large number of *Arabidopsis* SNAREs can be grouped by sequence similarity to yeast and animal counterparts involved in specific trafficking pathways, and some have been localized to the trans-Golgi and the pre-vacuolar pathway⁷⁷. *Arabidopsis* also has a receptor for retention of proteins in the endoplasmic reticulum, a cargo receptor for transport to the vacuole and several phragmoplastins related to animal dynamin GTPases. Thus, plant cells appear to use the same basic machinery for vesicle trafficking as yeast and animal cells.

Animal cells possess many functionally diverse small G-proteins of the Ras superfamily involved in signal transduction, AF reorganization, vesicle fusion and other processes. Surprisingly, *Arabidopsis* appears to lack genes for G-proteins of the Ras, Rho, Rac and Cdc42 subfamilies but has many Rab-type G-proteins involved in vesicle fusion and several Rop-type G-proteins, one of which has a role in actin organization of the tip-growing pollen tube⁷⁸. The significance of this divergent amplification of different subfamilies of small G-proteins in plants and animals remains to be determined.

Arabidopsis possesses cyclin-dependent kinases (CDKs), including a plant-specific Cdc2b kinase expressed in a cell-cycle-dependent manner, several cyclin subtypes, including a D-type cyclin that

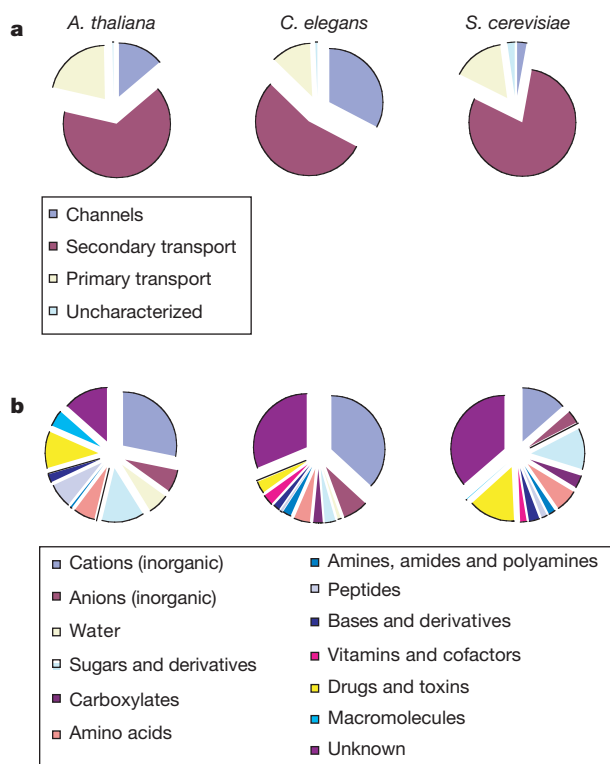


Figure 7 Comparison of the transport capabilities of *Arabidopsis*, *C. elegans* and *S. cerevisiae*. Pie charts show the percentage of transporters in each organism according to bioenergetics (a) and substrate specificity (b).

mediates cytokinin-stimulated cell-cycle progression⁷⁹, a retinoblastoma-related protein and components of the ubiquitin-dependent proteolytic pathway of cyclin degradation. In yeast and animal cells, chromosome condensation is mediated by condensins, sister chromatids are held together by cohesins such as Scc1, and metaphase–anaphase transition is triggered by separin/Esp1 endopeptidase proteolysis of Scc1 on APC-mediated degradation of its inhibitor, securin/Psd1. Related proteins are encoded by the *Arabidopsis* genome. Thus, the basic machinery of cell-cycle progression, genome duplication and segregation appears to be conserved in plants. By contrast, entry into M phase, M-phase progression and cytokinesis seem to be modified in plant cells. *Arabidopsis* does not appear to have homologues of Cdc25 phosphatase, which activates Cdc2 kinase at the onset of mitosis, or of polo kinase, which regulates M-phase progression in yeast and animals. Conversely, plant-specific mitogen-activated protein (MAP) kinases appear to be involved in cytokinesis.

Cytokinesis partitions the cytoplasm of the dividing cell. Yeast and animal cells expand the membrane from the surface towards the centre in a cleavage process supported by septins and a contractile ring of actin and type II myosin. By contrast, plant cytokinesis starts in the centre of the division plane and progresses laterally. A transient membrane compartment, the cell plate, is formed *de novo* by fusion of Golgi-derived vesicles trafficking along the phragmoplast MTs⁸⁰. Consistent with the unique mode of plant cytokinesis, *Arabidopsis* appears to lack genes for septins and type II myosin. Conversely, cell-plate formation requires a cytokinesis-specific syntaxin that has no close homologue in yeast and animals. Although syntaxin-mediated membrane fusion occurs in animal cytokinesis and cellularization, the vesicles are delivered to the base of the cleavage furrow. Thus, the plant-specific mechanism of cell division is linked to conserved eukaryotic cell-cycle machinery.

Two main conclusions are suggested by this comparative analysis. First, *Arabidopsis* and eukaryotic cells have common features related to intracellular activities, such as vesicle trafficking, cytoskeleton and cell cycle. Second, evolutionarily divergent features, such as organization of the cytoskeleton and cytokinesis, appear to relate to the plant cell wall.

Development

The regulation of development in *Arabidopsis*, as in animals, involves cell–cell communication, hierarchies of transcription factors, and the regulation of chromatin state; however, there is no reason to suppose that the complex multicellular states of plant and animal development have evolved by elaborating the same general processes during the 1.6 billion years since the last common unicellular ancestor of plants and animals^{81,82}. Our genome analyses reflect the long, independent evolution of many processes contributing to development in the two kingdoms.

Plants and animals have converged on similar processes of pattern formation, but have used and expanded different transcription factor families as key causal regulators. For example, segmentation in insects and differentiation along the anterior–posterior and limb axes in mammals both involve the spatially specific activation of a series of homeobox gene family members. The pattern of activation is causal in the later differentiation of body and limb axis regions. In plants the pattern of floral whorls (sepals, petals, stamens, carpels) is also established by the spatially specific activation of members of a family of transcription factors, but in this instance the family is the MADS box family. Plants also have homeobox genes and animals have MADS box genes, implying that each lineage invented separately its mechanism of spatial pattern formation, while converging on actions and interactions of transcription factors as the mechanism. Other examples show even greater divergence of plant and animal developmental control. Examples are the AP2/EREBP and NAC families of transcription factors, which have important roles in flower and meristem development; both families are so far found

only in plants (Supplementary Information Table 6).

A similar story can be told for cell–cell communication. Plants do not seem to have receptor tyrosine kinases, but the *Arabidopsis* genome has at least 340 genes for receptor Ser/Thr kinases, belonging to many different families, defined by their putative extracellular domains (Supplementary Information Table 7). Several families have members with known functions in cell–cell communication, such as the CLV1 receptor involved in meristem cell signalling, the S-glycoprotein homologues involved in signalling from pollen to stigma in self-incompatible *Brassica* species, and the BRI1 receptor necessary for brassinosteroid signalling⁸³. Animals also have receptor Ser/Thr kinases, such as the transforming growth factor- β (TGF- β) receptors, but these act through SMAD proteins that are absent from *Arabidopsis*. The leucine-rich repeat (LRR) family of *Arabidopsis* receptor kinases shares its extracellular domain with many animal and fungal proteins that do not have associated kinase domains, and there are at least 122 *Arabidopsis* genes that code for LRR proteins without a kinase domain. Other *Arabidopsis* receptor kinase families have extracellular domains that are unfamiliar in animals. Thus, evolution is modular, and the plant and animal lineages have expanded different families of receptor kinases for a similar set of developmental processes.

Several *Arabidopsis* genes of developmental importance appear to be derived from a cyanobacteria-like genome (Supplementary Information Table 2), with no close relationship to any animal or fungal protein. One salient example is the family of ethylene receptors; another gene family of apparent chloroplast origin is the phytochromes—light receptors involved in many developmental decisions (see below). Whereas the land plant phytochromes show clear homology to the cyanobacterial light receptors, which are typical prokaryotic histidine kinases, the plant phytochromes are histidine kinase paralogues with Ser/Thr specificity⁸⁴. Similarly to the ethylene receptors, the proteins that act downstream of plant phytochrome signalling are not found in cyanobacteria, and thus it appears that a bacterial light receptor entered the plant genome through horizontal transfer, altered its enzymatic activity, and became linked to a eukaryotic signal transduction pathway. This infusion of genes from a cyanobacterial endosymbiont shows that plants have a richer heritage of ancestral genes than animals, and unique developmental processes that derive from horizontal gene transfer.

Signal transduction

Being generally sessile organisms, plants have to respond to local environmental conditions by changing their physiology or redirecting their growth. Signals from the environment include light and pathogen attack, temperature, water, nutrients, touch and gravity. In addition to local cellular responses, some stimuli are communicated across the plant body, with plant hormones and peptides acting as secondary messengers. Some hormones, such as auxin, are taken up into the cell, whereas others, such as ethylene and brassinosteroids, and the peptide CLV3, act as ligands for receptor kinases on the plasma membrane. No matter where the signal is perceived by the cell, it is transduced to the nucleus, resulting in altered patterns of gene expression.

Comparative genome analysis between *Arabidopsis*, *C. elegans* and *Drosophila* supports the idea that plants have evolved their own pathways of signal transduction⁸⁵. None of the components of the widely adopted signalling pathways found in vertebrates, flies or worms, such as Wingless/Wnt, Hedgehog, Notch/lin12, JAK/STAT, TGF- β /SMADs, receptor tyrosine kinase/Ras or the nuclear steroid hormone receptors, is found in *Arabidopsis*. By contrast, brassinosteroids are ligands of the BRI1 Ser/Thr kinase, a member of the largest recognizable class of transmembrane sensors encoded by 340 receptor-like kinase (RLK) genes in the *Arabidopsis* genome (Supplementary Information Table 7). With a few notable exceptions, such as CLV1, the types of ligands sensed by RLKs are

completely unknown, providing an enormous future challenge for plant biologists. G-protein-coupled receptors (GPCRs)/ seven-transmembrane proteins are an abundant class of proteins in mammalian genomes, instrumental in signal transduction. INTERPRO detected 27 GPCR-related domains in *Arabidopsis* (Supplementary Information Table 1), although there is no direct experimental evidence for these. *Arabidopsis* contains a family of 18 seven-transmembrane proteins of the mildew resistance (MLO) class, several of which are involved in defence responses. Notably, only single G α (GPA1) and G β (AGB1) subunits are found in *Arabidopsis*, both previously known⁸⁶.

Although cyclic GMP has been proposed to be involved in signal transduction in *Arabidopsis*⁸⁷, a protein containing a guanylate cyclase domain was not identified in our analyses. Nevertheless, cyclic nucleotide-binding domains were detected in various proteins, indicating that cNMPs may have a role in plant signal transduction. Thus, although cNMP-binding domains appear to have been conserved during evolution, cNMP synthesis in *Arabidopsis* may have evolved independently.

We were unable to identify a protein with significant similarity to known G γ subunits, but recent biochemical studies suggest that a protein with this functional capacity is likely to be present in plant cells (H. Ma, personal communication). Therefore, there is potential for the formation of only a single heterotrimeric G-protein complex; however, its functional interaction with any of the potential GPCR-related proteins remains to be determined.

Modules of cellular signal pathways from bacteria and animals have been combined and new cascades have been innovated in plants. A pertinent example is the response to the gaseous plant hormone ethylene⁸⁸. Ethylene is perceived and its signal transmitted by a family of receptors related to bacterial-type two-component histidine kinases (HKs). In bacteria, yeast and plants, these proteins sense many extracellular signals and function in a His-to-Asp phosphorelay network⁸⁹. In turn, these proteins physically interact with the genetically downstream protein CTR1, a Raf/MAPKKK-related kinase, revealing the juxtaposition of bacterial-type two-component receptors and animal-type MAP kinase cascades. Unlike animals, however, *Arabidopsis* does not seem to have a Ras protein to activate the MAP kinase cascade. MAP kinases are found in abundance in *Arabidopsis*: we identified ~20, a higher number than in any other eukaryote. As potentially counteracting components, we found ~70 putative PP2C protein phosphatases. Although this group is largely uncharacterized functionally, several members are related to ABI1/ABI2, key negative regulators in the signalling pathway for the plant hormone abscisic acid. Additional components of the His-to-Asp phosphorelay system were also found in *Arabidopsis*, including authentic response regulators (ARRs), pseudoresponse regulators (PRRs) and phosphotransfer intermediate protein (HPT)⁹⁰. We found 11 HKs in the proteome (3 new), 16 RRs (2 new) and 8 PRRs (2 new). The biological roles of most ARRs, PRRs and HPTs are largely unknown, but several have been found to have diverse functions in plants, including transcriptional activation in response to the plant hormone cytokinin⁹¹, and as components of the circadian clock⁹².

Plants seem to have evolved unique signalling pathways by combining a conserved MAP kinase cascade module with new receptor types. In many cases, however, the ligands are unknown. Conversely, some known signalling molecules, such as auxin, are still in search of a receptor. Auxin signalling may represent yet another plant-specific mode of signalling, with protein degradation through the ubiquitin-proteasome pathway preceding altered gene expression. With many *Arabidopsis* genes encoding components of the ubiquitin-proteasome pathway, elimination of negative regulators may be a more widespread phenomenon in plant signalling.

Recognizing and responding to pathogens

Plants are constantly exposed to pests, parasites and pathogens and

have evolved many defences. In mammals, polymorphism for parasite recognition encoded in the MHC genes contributes to resistance. In plants, disease resistance (R) genes that confer parasite recognition are also extremely polymorphic. This polymorphism has been proposed to restrict parasites, and its absence may explain the breakdown of resistance in crop monocultures⁹³. In contrast to MHC genes, plant resistance genes are found at several loci, and the complete genome sequence enables analysis of their complement and structure. Parasite recognition by resistance genes triggers defence mechanisms through various signalling molecules, such as protein kinases and adapter proteins, ion fluxes, reactive oxygen intermediates and nitric oxide. These halt pathogen colonization through transcriptional activation of defence genes and a form of programmed cell death called the hypersensitive response⁹⁴. The *Arabidopsis* genome contains diverse resistance genes distributed at many loci, along with components of signalling pathways, and many other genes whose role in disease resistance has been inferred from mutant phenotypes.

Most resistance genes encode intracellular proteins with a nucleotide-binding (NB) site typical of small G proteins, and carboxy-terminal LRRs⁹⁵. Their amino termini either carry a TIR domain, or a putative coiled coil (CC). There are 85 TIR–NB–LRR resistance genes at 64 loci, and 36 CC–NB–LRR resistance genes at 30 loci. Some NB–LRR resistance genes express neither obvious TIR nor CC domains at their N termini. This potential class is present seven times, at six loci. There are 15 truncated TIR–NB genes that lack an LRR at 10 loci, often adjacent to full TIR–NB–LRR genes. There are also six CC–NB genes, at five loci. These truncated products may function in resistance. Intriguingly, two TIR–NB–LRR genes carry a WRKY domain, found in transcription factors that are implicated in plant defence, and one of these also encodes a protein kinase domain.

Resistance gene evolution may involve duplication and divergence of linked gene families³⁶; however, most (46) resistance genes are singletons; 50 are in pairs, 21 are in 7 clusters of 3 family members, with single clusters of 4, 5, 7, 8 and 9 members, respectively. Of the non-singletons, ~60% of pairs are in direct repeats, and ~40% are in inverted repeats. Resistance genes are unevenly distributed between chromosomes, with 49 on chromosome 1; 2 on chromosome 2; 16 on chromosome 3; 28 on chromosome 4; and 55 on chromosome 5.

In other plant species, resistance genes encode both transmembrane receptors for secreted pathogen products and protein kinases, and some other classes are also found. The *Cf* genes in tomato encode extracellular LRRs with a transmembrane domain and short cytoplasmic domain. Mutation in an *Arabidopsis* homologue, *CLAVATA2*, results in enlarged meristems, but to date no resistance function has been assigned to the 30 *Arabidopsis* *CLV2* homologues. *CLAVATA1*, a transmembrane LRR kinase, is also required for meristem function. *Xa21*, a rice LRR-kinase, confers *Xanthomonas* resistance, and the *Arabidopsis* *FLS2* LRR kinase confers recognition of flagellin. It has been proposed that CLV1 and CLV2 function as a heterodimer; perhaps this is also true for *Xa21*, *FLS2* and *Cf* proteins. There are 174 LRR transmembrane kinases in *Arabidopsis*, with only *FLS2* assigned a role in resistance. A unique resistance gene, beet *Hs1pro-1*, which confers nematode resistance, has two *Arabidopsis* homologues.

The tomato Pto Ser/Thr kinase acts as a resistance protein in conjunction with an NB–LRR protein, so similar kinases might do the same for *Arabidopsis* NB–LRR proteins. There are 860 Ser/Thr kinases in the *Arabidopsis* sequence. Fifteen of these share 50% identity over the Pto-aligned region. The Toll pathway in *Drosophila* and mammals regulates innate immune responses through LRR/TIR domain receptors that recognize bacterial lipopolysaccharides⁹⁶. Pto is highly homologous to *Drosophila* PELLE and mammalian IRAK protein kinases that mediate the TIR pathway.

Additional genes have been defined that are required for resistance by our analysis of the genome sequence. The *ndr1* mutation defines a gene required by the CC–NB–LRR gene *RPS2* and *RPM1*. *NDR1* is 1 of 28 *Arabidopsis* genes that are similar both to each other and to the tobacco *HIN1* gene that is transcriptionally induced early during the hypersensitive response. *EDS1* is a gene required for TIR–NB–LRR function, and like *PAD4*, encodes a protein with a putative lipase motif. *EDS1*, *PAD4* and a third gene comprise the *EDS1/PAD4* family. The *NPR1/NIM1/SAI1* gene is required for systemic acquired resistance, and we found five additional *NPR1* homologues. Recessive mutations at both the barley Mlo and *Arabidopsis* *LSD1* loci confer broad-spectrum resistance and derepress a cell-death program. There are at least 18 Mlo family members that resemble heterotrimeric GPCRs in *Arabidopsis*, and only two *LSD1* homologues.

One of the earliest responses to pathogen recognition is the production of reactive oxygen intermediates. This involves a specialized respiratory burst oxidase protein that transfers an electron across the plasma membrane to make superoxide. *Arabidopsis* encodes eight apparently functional *gp91* homologues, called *Atrboh* genes. Unlike *gp91*, they all carry an ~300 amino-acid N-terminal extension carrying an EF-hand Ca^{2+} -binding domain. In mammals, activation of the respiratory oxidative burst complex in the neutrophil, which includes *gp91*, requires the action of Rac proteins. As no Rac or Ras proteins are found in *Arabidopsis*, members of the large *rop* family of G proteins may carry this out. Similarly, we did not detect any *Arabidopsis* homologues of other mammalian respiratory burst oxidase components (p22, p47, p67, p40).

There are no clear homologues of many mammalian defence and cell-death control genes. Although nitric oxide production is involved in plant defence, there is no obvious homologue of nitric oxide synthase. Also absent are apparent homologues of the REL domain transcription factors involved in innate immunity in both *Drosophila* and mammals. We found no similarity to proteins involved in regulating apoptosis in animal cells, such as classical caspases, *bcl2/ced9* and baculovirus p35. There are, however, 36 cysteine proteases. There are also eight homologues of a newly defined metacaspase family⁹⁷, two of which, along with *LSD1*, have a clear GATA-type zinc-finger.

Photomorphogenesis and photosynthesis

Because nearly all plants are sessile and most depend on photosynthesis, they have evolved unique ways of responding to light. Light serves as an energy source, as well as a trigger and modulator of complex developmental pathways, including those regulated by the circadian clock. Light is especially important during seedling emergence, where it stimulates chlorophyll production, leaf development, cotyledon expansion, chloroplast biogenesis and the coordinated induction of many nuclear- and chloroplast-encoded genes, while at the same time inhibiting stem growth. The goal of this process, called photomorphogenesis, is the establishment of a body plan that allows the plant to be an efficient photosynthetic machine under varying light conditions⁹⁸. The signal transduction cascade leading to light-induced responses begins with the activation of photoreceptors. Next, the light signal is transduced via positively and negatively acting nuclear and cytoplasmic proteins, causing activation or derepression of nuclear and chloroplast-encoded photosynthetic genes and enabling the plant to establish optimal photoautotrophic growth. Although genetic and biochemical studies have defined many of the components in this process, the genome sequence provides an opportunity to identify comprehensively *Arabidopsis* genes involved in photomorphogenesis and the establishment of photoautotrophic growth. We identified at least 100 candidate genes involved in light perception and signalling, and 139 nuclear-encoded genes that potentially function in photosynthesis.

The roles have been described of only 35 of the 100 candidate photomorphogenic genes (Supplementary Information Table 8). All of the light photoreceptors had been discovered previously, including five red/far-red absorbing phytochromes (PHYA–E), two blue/ultraviolet-A absorbing cryptochromes (CRY1 and CRY2), one blue-absorbing phototropin (NPH1) and one NPH1-like (or NPL1). In contrast, we uncovered many new proteins similar to the photomorphogenesis regulators COP/DET/FUS, PKS1, PIF3, NDPK2, SPA1, FAR1, GIGANTEA, FIN219, HY5, CCA1, ATHB-2, ZEITLUPE, FKF1, LKP1, NPH3 and RPT2.

Both the phytochromes and NPH1 contain chromophores for light sensing coupled to kinase domains for signal transmission. Phytochromes have an N-terminal chromophore-binding domain, two PAS domains, and a C-terminal Ser/Thr kinase domain⁹⁹, whereas NPH1 has two LOV domains (members of the PAS domain superfamily) for flavin mononucleotide binding and a C-terminal Ser/Thr kinase domain¹⁰⁰. PAS domains potentially sense changes in light, redox potential and oxygen energy levels, as well as mediating protein–protein interactions^{99,100}. We searched for uncharacterized proteins with the combination of a kinase domain and either a phytochrome chromophore-binding site or PAS domains. Although we found no new phytochrome-like genes, we did identify four predicted proteins that contain PAS and kinase domains (Supplementary Information Fig. 6). These proteins share 80% amino-acid identity, but, unlike NPH1 and NPL1, have only one PAS domain. The combination of potential signal sensing and transmitting domains makes it tempting to speculate that these proteins may be receptors for light or other signals.

Our screen included searches for components of photosynthetic reaction centres and light-harvesting complexes, enzymes involved in CO_2 fixation and enzymes in pigment biosynthesis. We identified 11 core proteins of photosystem I, including the eukaryotic-specific components PsuG and PsuH¹⁰¹, and 8 photosystem II proteins, including a single member (*psbW*) of the photosystem II core. We also found 26 proteins similar to the Chlorophyll-a/b binding proteins (8 Lhca and 18 Lhcb). Of the seven subunits of the cytochrome *b₆f* complex (*PetA–D*, *PetG*, *PetL*, *PetM*), only one (*PetC*) was found in the nuclear genome, whereas the remainder are probably encoded in the chloroplast. Similarly, of the nine subunits of the chloroplast ATP synthase complex, three are encoded in the nucleus, including the II-, γ - and δ -subunits; the remaining subunits (I, III, IV, α , β , ϵ) are encoded in the chloroplast¹⁰². Ten genes were related to the soluble components of the electron transfer chain, including two plastocyanins, five ferredoxins and three ferredoxin/NADP oxidoreductases. Forty genes are predicted to have a role in CO_2 fixation, including all of the enzymes in the Calvin–Benson cycle. For pigment biosynthesis, 16 genes in chlorophyll biosynthesis and 31 genes in carotenoid biosynthesis were found (Supplementary Information Table 8). Our analyses have identified several potential components of the light perception pathway, and have revealed the complex distribution of components of the photosynthetic apparatus between nuclear and plastid genomes.

Metabolism

Arabidopsis is an autotrophic organism that needs only minerals, light, water and air to grow. Consequently, a large proportion of the genome encodes enzymes that support metabolic processes, such as photosynthesis, respiration, intermediary metabolism, mineral acquisition, and the synthesis of lipids, fatty acids, amino acids, nucleotides and cofactors¹⁰³. With respect to these processes, *Arabidopsis* appears to contain a complement of genes similar to those in the photoautotrophic cyanobacterium *Synechocystis*⁴⁵, but, whereas *Synechocystis* generally has a single gene encoding an enzyme, *Arabidopsis* frequently has many. For example, *Arabidopsis* has at least seven genes for the glycolytic enzyme pyruvate kinase, with an

additional five for pyruvate kinase-like proteins. Whatever the reason for this high level of redundancy, it varies from gene to gene in the same pathway; the 11 enzymes of glycolysis are encoded by up to 51 genes that are present in as few as one or as many as eight copies. Similarly, of the 59 genes encoding proteins involved in glycerolipid metabolism, 39 are represented by more than one gene¹⁰⁴. Genome duplication and expansion of gene families by tandem duplication have contributed to this diversity.

This high degree of apparent structural redundancy does not necessarily imply functional redundancy. For instance, although there are seven genes for serine hydroxymethyltransferase, a mutation in the gene for the mitochondrial form completely blocks the photorespiratory pathway¹⁰⁵. Although there are 12 genes for cellulose synthase, mutations in at least 2 of the 12 confer distinct phenotypes because of tissue-specific gene expression¹⁰⁶.

The metabolome of *Arabidopsis* differs from that of cyanobacteria, or of any other organism sequenced to date, by the presence of many genes encoding enzymes for pathways that are unique to vascular plants. In particular, although relatively little is known about the enzymology of cell-wall metabolism, more than 420 genes could be assigned probable roles in pathways responsible for the synthesis and modification of cell-wall polymers. Twelve genes encode cellulose synthase, and 29 other genes encode 6 families of structurally related enzymes thought to synthesize other major polysaccharides¹⁰⁶. Roughly 52 genes encode polygalacturonases, 20 encode pectate lyases and 79 encode pectin esterases, indicating a massive investment in modifying pectin. Similarly, the presence of 39 β -1,3-glucanases, 20 endoxylglucan transglycosylases, 50 cellulases and other hydrolases, and 23 expansins reflects the importance of wall remodelling during growth of plant cells. Excluding ascorbate and glutathione peroxidases, there are 69 genes with significant similarity to known peroxidases and 15 laccases (diphenol oxidases). Their presence in such abundance indicates the importance of oxidative processes in the synthesis of lignin, suberin and other cell-wall polymers. The high degree of apparent redundancy in the genes for cell-wall metabolism might reflect differences in substrate specificity by some of the enzymes.

The high degree of apparent redundancy in the genes for cell wall metabolism might reflect differences in substrate specificity by some of the enzymes. It is already known that cell types have different wall compositions, which may require that the relevant enzymes be subject to cell-type-specific transcriptional regulation. Of the 40 or so cell types that plants make, almost all can be identified by unique features of their cell wall¹⁰⁷. A large number of genes involved in wall metabolism have yet to be defined. Although more than 60 genes for glycosyltransferases can be found in the genome sequence, most of these are probably involved in protein glycosylation or metabolite catabolism and do not seem to be adequate to account for the polysaccharide complexity of the wall. For instance, at least 21 enzymes are required just to produce the linkages of the pectic polysaccharide RGII, and none of these enzymes has been identified at present. Thus, if these and related enzymes involved in the synthesis of other cell-wall polymers are also represented by multiple genes, a substantial number of the genes of currently unknown function may be involved in cell-wall metabolism.

Higher plants collectively synthesize more than 100,000 secondary metabolites. Because flowering plants are thought to have similar numbers of genes, it is apparent that a great deal of enzyme creation took place during the evolution of higher plants. An important factor in the rapid evolution of metabolic complexity is the large family of cytochrome P450s that are evident in *Arabidopsis* (Supplementary Information Table 1). These enzymes represent a superfamily of haem-containing proteins, most of which catalyse NADPH- and O₂-dependent hydroxylation reactions. Plant P450s participate in myriad biochemical pathways including those devoted to the synthesis of plant products, such as phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides and

glucosinolates, and plant growth regulators, such as gibberellins, jasmonic acid and brassinosteroids. Whereas *Arabidopsis* has ~286 P450 genes, *Drosophila* has 94, *C. elegans* has 73 and yeast has only 3. This low number in yeast indicates that there are few reactions of basic metabolism that are catalysed by P450s. It seems likely that many animal P450s are involved in detoxification of compounds from food plant sources. The role of endogenous enzymes is poorly understood; only a few dozen P450 enzymes from plants have been characterized to any extent. The discrepancy between the number of known P450-catalysed reactions and the number of genes suggests that *Arabidopsis* produces a relatively large number of metabolites that have yet to be identified.

In addition to the large number of cytochrome P450s, *Arabidopsis* has many other genes that suggest the existence of pathways or processes that are not currently known. For instance, the presence of 19 genes with similarity to anthranilate *N*-hydroxycinnamoyl/benzoyl transferase is currently inexplicable. This enzyme is involved in the synthesis of dianthramide phytoalexins in Caryophyllaceae and Gramineae. No phytoalexins of this class have been described in *Arabidopsis* as yet. Similarly, the presence of 12 genes with sequence similarity to the berberine bridge enzyme, ((S)-reticuline:oxygen oxidoreductase (methylene-bridge-forming); EC 1.5.3.9), and 13 genes with similarity to tropinone reductase, suggests that *Arabidopsis* may have the ability to produce alkaloids. In other plants, the berberine bridge enzyme transforms reticuline into scoulerine, a biosynthetic precursor to a multitude of species-specific protopine, protoberberine and benzophenanthridine alkaloids. The discovery of these and many other intriguing genes in the *Arabidopsis* genome has created a wealth of new opportunities to understand the metabolic and structural diversity of higher plants.

Concluding remarks

The twentieth century began with the rediscovery of Mendel's rules of inheritance in pea¹⁰⁸, and it ends with the elucidation of the complete genetic complement of a model plant, *Arabidopsis*. The analysis of the completed sequence of a flowering plant reported here provides insights into the genetic basis of the similarities and differences of diverse multicellular organisms. It also creates the potential for direct and efficient access to a much deeper understanding of plant development and environmental responses, and permits the structure and dynamics of plant genomes to be assessed and understood.

Arabidopsis, *C. elegans* and *Drosophila* have a similar range of 11,000–15,000 different types of proteins, suggesting this is the minimal complexity required by extremely diverse multicellular eukaryotes to execute development and respond to their environment. We account for the larger number of gene copies in *Arabidopsis* compared with these other sequenced eukaryotes with two possible explanations. First, independent amplification of individual genes has generated tandem and dispersed gene families to a greater extent in *Arabidopsis*, and unequal crossing over may be the predominant mechanism involved. Second, ancestral duplication of the entire genome and subsequent rearrangements have resulted in segmental duplications. The pattern of these duplications suggests an ancient polyploidy event, and mutant analysis indicates that at least some of the many duplicate genes are functionally redundant. Their occurrence in a functionally diploid genetic model came as a surprise, and is reminiscent of the situation in maize, an ancient segmental allotetraploid. The remarkable degree of genome plasticity revealed in the large-scale duplications may be needed to provide new functions, as alternative promoters and alternative splicing appear to be less widely used in plants than they are in animals. Apart from duplicated segments, the overall chromosome structure of *Arabidopsis* closely resembles that of *Drosophila*; transposons and other repetitive sequences are concentrated in the heterochromatic regions surrounding the centromere,

whereas the euchromatic arms are largely devoid of repetitive sequences. Conversely, most protein-coding genes reside in the euchromatin, although a number of expressed genes have been identified in centromeric regions. Finally, *Arabidopsis* is the first methylated eukaryotic genome to be sequenced, and will be invaluable in the study of epigenetic inheritance and gene regulation.

Unlike most animals, plants generally do not move, they can perpetuate indefinitely, they reproduce through an extended haploid phase, and they synthesize all their metabolites. Our comparison of *Arabidopsis*, bacterial, fungal and animal genomes starts to define the genetic basis for these differences between plants and other life forms. Basic intracellular processes, such as translation or vesicle trafficking, appear to be conserved across kingdoms, reflecting a common eukaryotic heritage. More elaborate intercellular processes, including physiology and development, use different sets of components. For example, membrane channels, transporters and signalling components are very different in plants and animals, and the large number of transcription factors unique to plants contrasts with the conservation of many chromatin proteins across the three eukaryotic kingdoms. Unexpected differences between seemingly similar processes include the absence of intracellular regulators of cell division (Cdc25) and apoptosis (Bcl-2). On the other hand, DNA repair appears more highly conserved between plants and mammals than within the animal kingdom, perhaps reflecting common factors such as DNA methylation. Our analysis also shows that many genes of the endosymbiotic ancestor of the plastid have been transferred to the nucleus, and the products of this rich prokaryotic heritage contribute to diverse functions such as photoautotrophic growth and signalling.

The sequence reported here changes the fundamental nature of plant genetic analysis. Forward genetics is greatly simplified as mutations are more conveniently isolated molecularly, but at the same time extensive gene duplications mean that functional redundancy must be taken into account. At a biochemical level, the specificity conferred by nucleotide sequence, and the completeness of the survey allow complex mixtures of RNA and protein to be resolved into their individual components using micro-arrays and mass spectrometry. This specificity can also be used in the parallel analysis of genome-wide polymorphisms and quantitative traits in natural populations¹⁰⁹. Looking ahead, the challenge of determining the function of the large set of predicted genes, many of which are plant-specific, is now a clear priority, and multinational programs have been initiated to accomplish this goal using site-selected mutagenesis among the necessary tools¹¹⁰. Finally, productive paths of crop improvement, based on enhanced knowledge of *Arabidopsis* gene function, will help meet the challenge of sustaining our food supply in the coming years.

Note added in proof: at the time of publication 17 centromeric BACs and 5 sequence gaps in chromosome arms are being sequenced. □

Methods

The three centres used similar annotation approaches involving in silico gene-finding methods, comparison to EST and protein databases, and manual reconciliation of that data. Gene finding involved three steps: (1) analysis of BAC sequences using a computational gene finder; (2) alignment of the sequence to the protein and EST databases; (3) assignment of functions to each of the genes. Genscan¹¹¹, GeneMark.HMM¹¹², Xgrail¹¹³ Genefinder (P. Green, unpublished software) and GlimmerA¹¹⁴ were used to analyse BAC sequences. All of these systems were specially trained for *Arabidopsis* genes. Splice sites were predicted using NetGene2¹¹⁵, Splice Predictor¹¹⁶ and GeneSplicer (M. Pertea and S. Salzberg, unpublished software). For the second step, BACs were aligned to ESTs and to the *Arabidopsis* gene index¹¹⁷ using programs such as DDS/GAP2¹¹⁸ or BLASTN¹¹⁹. Segmental duplications were analysed and displayed using a modified version of DIALIGN2 (ref. 120).

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Sequence and analysis of chromosome 1 of the plant *Arabidopsis thaliana*

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The genome of the flowering plant *Arabidopsis thaliana* has five chromosomes^{1,2}. Here we report the sequence of the largest, chromosome 1, in two contigs of around 14.2 and 14.6 megabases. The contigs extend from the telomeres to the centromeric borders, regions rich in transposons, retrotransposons and repetitive elements such as the 180-base-pair repeat. The chromosome represents 25% of the genome and contains about 6,850 open reading frames, 236 transfer RNAs (tRNAs) and 12 small nuclear RNAs. There are two clusters of tRNA genes at different places on the chromosome. One consists of 27 tRNA^{Pro} genes and the other contains 27 tandem repeats of tRNA^{Tyr}-tRNA^{Tyr}-tRNA^{Ser} genes. Chromosome 1 contains about 300 gene families with clustered duplications. There are also many repeat elements, representing 8% of the sequence.

Future insights into basic plant biology and the ability to manipulate plants through genetic engineering for agronomic improvement will depend on whether we can identify the genes that control fundamental developmental and metabolic processes. The identification of these genes has until recently relied on their mutant phenotypes, genetic map positions and positional

cloning approaches. Sequencing the complete genomes of model organisms³⁻⁵ has greatly facilitated the identification of genes that are important for both basic biological and disease-related processes. Most plant species have relatively large genomes (>500 megabases (Mb)) with many repeated sequences. Complete genome sequencing efforts in plants have therefore centred largely on *Arabidopsis thaliana*^{6,7}, which has a small genome (around 130 Mb) with low repetitive DNA content^{2,8,9}. The Arabidopsis Genome Initiative (AGI) was established in 1996 to sequence the genome of *Arabidopsis*. Here we report the DNA sequence of 28.8 Mb of chromosome 1 of ecotype Columbia, fully annotated into two contigs—the northern arm (approximately 14.2 Mb) and the southern arm (approximately 14.6 Mb).

Chromosome 1 is metacentric and was originally estimated, using the yeast artificial chromosome (YAC)-based physical map¹⁰, to be 31-Mb long. Sequencing was initiated using bacterial artificial chromosomes (BACs) anchored to a physical map¹¹. An optimal tiling path for sequencing was determined using a BAC end-sequencing strategy¹². Three AGI groups, Genoscope (<http://www.genoscope.cns.fr/externe/arabidopsis>), The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/at/abe/bac_end_search.html) and the Stanford/University of Pennsylvania/Plant Gene Expression Center (SPP) Consortium (<http://genome.bio.upenn.edu/physical-mapping/bacendlist/bacends.html>) determined the end sequences for almost all of the BACs in the TAMU¹³ and

Table 1 Features of chromosome 1

Feature	Value	
(a) The DNA molecule		
Length	28,762,046 bp	
Top arm	14,172,442 bp	
Bottom arm	14,589,604 bp	
Base composition (% GC)		
Overall	35.8	
Coding	43.8	
Non-coding	31.8	
Number of genes	6,848	
Gene density	4.1 kb per gene	
Average gene length	2,145 bp	
Average peptide length	460 amino acids	
Exons		
Number	35,768	
Total length	9,396,363 bp (33%)	
Average per gene	5.2	
Average size	272 bp	
Introns		
Number	28,951	
Total length	4,807,531 bp (17%)	
Average size	166	
Number of genes with ESTs	3,448 (50%)	
Number of ESTs* for chromosome 1 genes	~30,000 (27%)	
(b) The proteome		
Classification/function	Number	Percentage (%)
Total proteins	6,848	100
With similarity to GenBank entries	4,793	70
Unknown	1,590	23
Hypothetical	1,935	28
With putative function	3,323	49
With putative signal peptides		
Secretory pathway	1,049	15
Chloroplast	521	7.5
Mitochondria	96	1.5
Classification of proteins with putative function		
Cellular metabolism	995	30
Transcription	498	15
Plant defence	354	11
Signalling	327	10
Growth and development	302	9
Protein fate	292	9
Intracellular transport	247	7
Ion transport	194	6
Protein synthesis	113	3
Total	3,322	100

* Around 110,000 ESTs used for analysis (<http://www.arabidopsis.org>).

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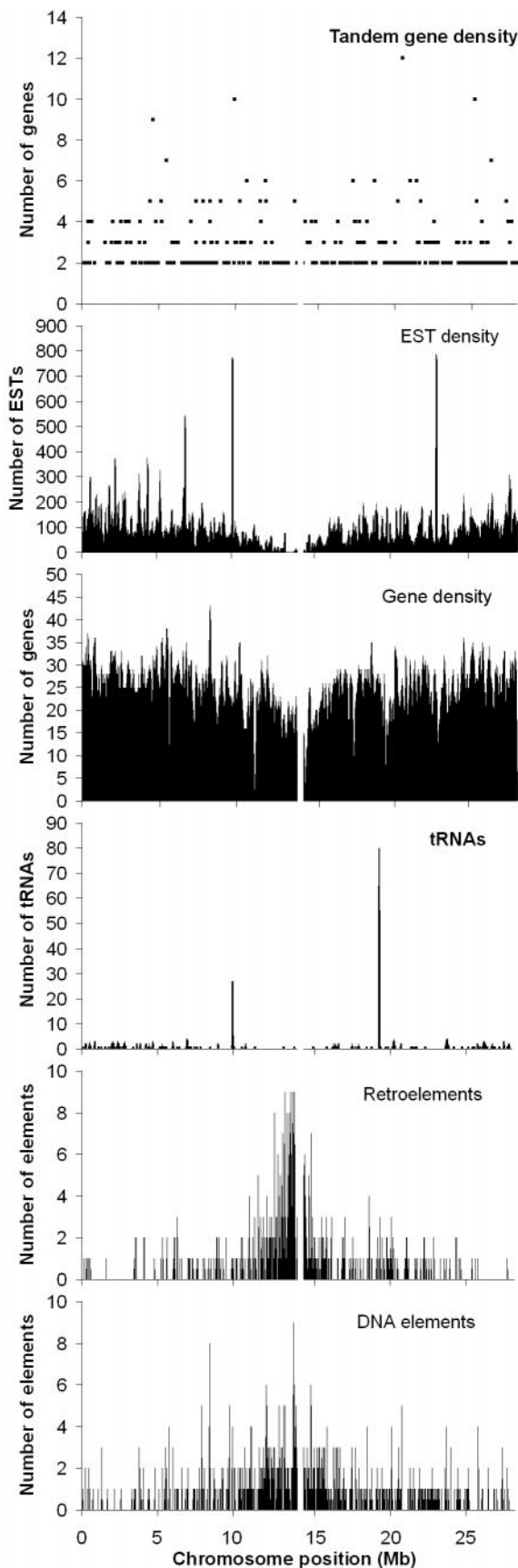


Figure 1 Density of various features along chromosome 1. The analyses were performed on the entire chromosome (28,762,046 bp) by joining the two arms at the centromeric gap, shown as a discontinuity. The densities of the features were obtained by analysing the sequence every 100 kb using a 10-kb sliding window. The analyses for retro- and DNA-transposable elements were carried out every 10 kb.

IGF¹⁴ libraries, corresponding to about 36,000 unique end sequences from around 18,300 BAC clones (~14× coverage; 10,200 TAMU and 8,100 IGF clones). We searched completed BAC sequences against the end sequence database, and chose BAC clones with a minimum of sequence overlap for further sequencing. On average, we identified homologous BAC end sequences every five kilobases (kb) along the length of the query BAC sequence. We sequenced one YAC and 369 BAC clones to produce the finished unique sequence. Seventy-seven clones served as 'seed' points. The two contigs extend from the telomeres to the centromeric borders, which are rich in transposons, retrotransposons and diverse repetitive elements¹⁵. We identified the telomeric regions of the chromosome with the aid of previously isolated putative north (pAtT60) and south (ATYpATT1) telomeric clones from the Landsberg ecotype¹⁶ as described (see Methods in the Supplementary Information). The GC content of the chromosome is low, which made it easier to sequence using the available chemistry (Table 1(a)). The northern arm does not have any sequencing gaps. The southern arm has three sequencing gaps at 15.349 Mb (BAC T18F15, C084807), 15.573 Mb (BAC T2P3, C084820), and 20.070 Mb (BAC T4M14, AC027036).

We verified the collinearity of the assembled sequence using BLAST¹⁷ analysis of the BAC end sequences against the assembled contig sequences. In addition, the location of 107 sequenced chromosome 1 markers on the assembled chromosome is collinear with their location on the recombinant inbred linkage map¹⁸. The sequence accuracy is estimated to be no more than one error in 25,000 bp, on the basis of the discrepancies found in the sequenced overlapping regions of BACs. The centromere is estimated to be 1.3-Mb long (refs 15, 19), bringing the overall size of the chromosome to about 30 Mb. The relationship between physical and genetic distance is around 230 kb cM⁻¹, uniform across the chromosome and similar to that reported for chromosomes 2 (ref. 6) and 4 (ref. 7).

We used gene prediction programs and database searches to annotate and determine some of the features of the chromosome. It contains about 6,850 putative genes at a density of 1 gene per 4.1 kb, which is more than twice the density found in *Drosophila*⁵. The average gene is about 2-kb long (from start to stop codon) and contains five exons with an average size of 272 base pairs (bp). Therefore, almost 50% of the chromosome consists of genes (Table 1(a)). Approximately 1,250 (18%) of the genes are intronless and many have been annotated as 'hypothetical' proteins. The largest gene, F5A8.4 (AC004146), contains 68 exons and encodes a 5,139-amino-acid polypeptide (relative molecular mass ~500,000 (M_r ~500K) which is a ubiquitous putative membrane protein found in *Saccharomyces cerevisiae*³, *Caenorhabditis elegans*⁴, *D. melanogaster*⁷ and *Homo sapiens*. The smallest recognized gene, T6H22.15 (AC009894), encodes the L41 protein of the 60S ribosomal complex, which contains 25 amino acids. Expressed sequence tags (ESTs) exist for this gene. At least 3,448 (50%) genes are expressed, as shown by the presence of corresponding *Arabidopsis* complementary DNAs or ESTs in GenBank. Thirty-thousand (27%) of the 110,000 ESTs screened are products of chromosome 1 genes (Table 1(a)). Ninety-seven per cent of the chromosome 1 ESTs map to annotated genes. Genes that are most highly represented by ESTs include putative chlorophyll a/b-binding protein, small subunit of RuBP carboxylase, ferredoxin/precursor and photosystem II 10K polypeptide. The gene density and EST distribution are more or less the same along the chromosome, with their lowest values around the regions flanking the centromere. There are a few highly dense EST regions with more than 700 ESTs per 100 kb, indicating highly transcribed regions (Fig. 1).

The chromosome contains 236 tRNAs, which is similar to the total number (293) found in the *Drosophila* genome⁵. The tRNAs are evenly distributed along the chromosome except for two regions where they cluster (Fig. 1). The first cluster (at 9.989 Mb) contains 27 tandem tRNA^{Pro} genes, with the last gene being a pseudogene.

The other (at 19.282 Mb) has 27 tandem repeats of the tri-repeat tRNA^{Tyr}-tRNA^{Tyr}-tRNA^{Ser} (Fig. 2). The tRNA^{Ser}/tRNA^{Tyr} cluster has been structurally characterized²⁰ using conventional molecular approaches, but the tRNA^{Pro} cluster was previously undetected. The sequence homology among the tRNA repeats ranges from 98% to 99%. The three tRNA genes have the same orientation and are separated by 250 and 370 bp, respectively. It has been suggested that tRNA clustering may reflect tissue-specific co-regulation of the tRNA genes²⁰.

There are four pairs of megabase-scale segmental duplications within the chromosome. Their locations are: 3.1–3.8/19.3–19.8; 5.6–6/27.8–27.3; 6.2–7.5/25.4–26.7; and 8–8.6/25–24.3 Mb. The first and third duplications have the same orientation relative to the centromere; for the second and fourth set, the two members are in opposite orientations. There are also large-scale duplications between chromosome 1 and each of the other *Arabidopsis* chromosomes¹⁹. Analysis also reveals diverse repetitive elements representing 8% of the sequence (see Table 2 in the Supplementary Information) consisting of various retroelements (2.6%), DNA elements (2.4%) and a number of simple and low-complexity repeats (2.6%). There is an inverse relationship between gene density and retro- and DNA-element densities in the borders of the centromeric region (Fig. 1), a hallmark of such chromosomal domains²¹.

Computational analysis predicts 6,848 proteins (Table 1(b)), corresponding to about a quarter of the *Arabidopsis* proteome and equal to half the predicted number of proteins in *Drosophila* (13,601 predicted proteins). Around one-third (28%) of the proteins are 'hypothetical', being predicted by various gene prediction programs, but without EST matches. Twenty-five per cent have an 'unknown' function, but we know that they are transcriptionally active because there is an EST. Seventy per cent of the annotated proteins have some similarity to other 'hypothetical', 'unknown' or 'putative function' proteins from plants and other eukaryotic genomes such as *S. cerevisiae*³, *C. elegans*⁴, *D. melanogaster*⁵ and *H. sapiens*. Table 1(b) shows a functional classification of the proteins, based on their amino-acid motifs. Thirty per cent of the proteins with putative function participate in cellular metabolism, and another 50% are involved in transcription, plant defence, signalling, and growth and development. Comparison of the predicted proteins of chromosome 1 with other proteins from available complete genome sequences reveals that more than 1,500 proteins have significant homology (cutoff $\leq 10^{-30}$) to proteins from the

available genomes (*S. cerevisiae*, *C. elegans*, *D. melanogaster* and *H. sapiens*). The top 14 homologies are shown in Table 3 of the Supplementary Information. Clearly, proteins involved in RNA splicing, tRNA biosynthesis, translation and metabolism are highly conserved throughout the eukaryotic kingdom.

The chromosome contains 312 families of tandemly arranged genes with 847 members (comprising 12% of all the genes that have a blast score of more than 200). There are between two and twelve members in each family (Fig. 3). These families are distributed throughout the chromosome (Fig. 1). One hundred and fifty-six families encode a set of distinct protein isoforms and are not members of superfamilies. The remaining 156 families are members of 46 superfamilies, which vary in size from 2 to 23 families. For example, the T28K15.6 (AC022522) superfamily contains five two-member families, which encode putative NBS/LRR disease-resistance proteins. Two of the T28K15.6 families are located at ~4 Mb and the other three at ~20.5 Mb on the chromosome. The families

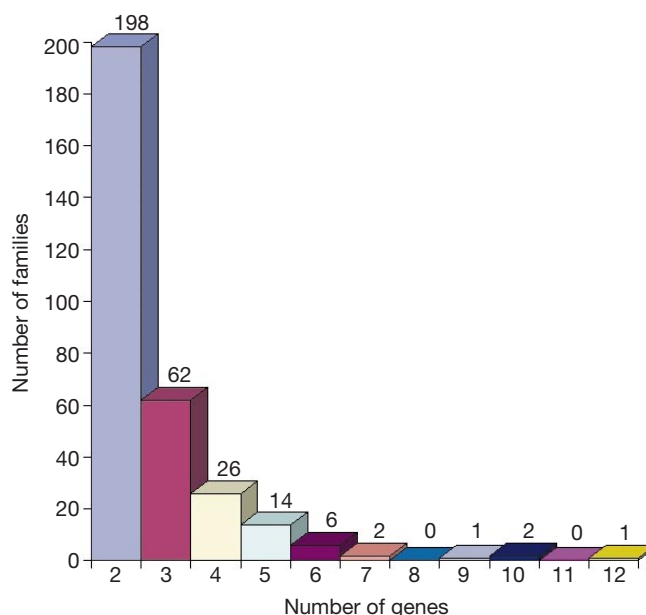


Figure 3 Frequency distribution of genes in multigene families with tandem gene arrangements.

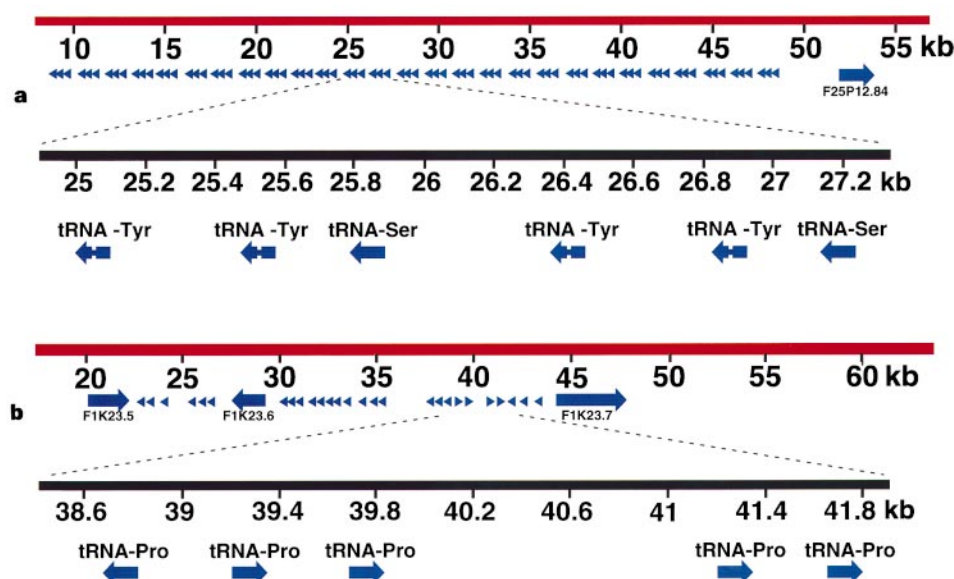


Figure 2 Clusters of tRNA genes in chromosome 1. **a**, The tRNA^{Ser}/tRNA^{Tyr} repeat cluster at 19.282 Mb is located on BAC F25P12 (accession no. AC009323). **b**, The tRNA^{Pro} cluster at 9.989 Mb is located on BAC F1K23 (accession no. AC007508).

of 25 superfamilies are close together (less than 2 Mb apart), whereas those of the remaining 21 are farther apart. In a few cases, the families are 20 Mb apart. For example, the T28P6.7 (AC007259) superfamily, which encodes S-locus-like receptor kinases (SRKs), has four families with two, three, twelve and two members located at 3.79, 38.1, 20.5 and 22.33 Mb, respectively. The first two SRK families are closely linked, separated by two unrelated genes. The SRK proteins have been implicated in controlling self-incompatibility in the reproductive system in *Brassicaceae*²², and it has been proposed that the autogamous reproductive system of *Arabidopsis* reflects the absence of the SRK genes from the *Arabidopsis* genome²². It remains to be determined whether the 19 putative SRK isoforms encoded by the T28P6.7 superfamily and the 12 SRK isoforms encoded by the T28P6.2 superfamily (three families with three, seven and two members, respectively) on chromosome 1 have an S-locus protein kinase activity. The largest superfamily is F22L4.6 (AC061957) with 23 families, which encodes 78 isoforms of a putative protein kinase. A large percentage of the gene families on this chromosome encode proteins that are involved in disease resistance, cell wall degradation and secondary metabolite biosynthesis.

It will be important to find out the biological significance of the multigene families. Why do so many gene products encode isoforms of the same polypeptide? This question applies to gene families with tandem gene arrangement as well as those with dispersed gene arrangements, such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS). This family has two members (ACS2 and ACS10) on chromosome 1 and eight other members in the other four chromosomes. It has been suggested²³ that the presence of different ACS isoforms may reflect tissue-specific expression that satisfies the biochemical properties of the cells or tissues in which each is expressed. For example, if a group of cells or tissues have low concentrations of the substrate, S-adenosyl methionine, then these cells express a high affinity (low Km) ACS isoform. Accordingly, the distinct biological function of each isoform is defined by its biochemical properties, which in turn define its tissue-specific expression. Such a concept can accommodate gene families encoding structural proteins as well as enzymes.

The sequences of chromosome 1 and the other *Arabidopsis* chromosomes^{6,7,24,25} will provide a wealth of information, but more experimental work is required to determine gene functions that will advance plant science²⁶. Most of the genes and their predicted functions should be interpreted with caution. Mapping the transcriptional units of the *Arabidopsis* chromosomes in the future will provide experimental verification of the annotation. Chip technology²⁷ has the potential to facilitate the mapping of the transcriptional units, leading to the isolation of full-length cDNA clones for all the genes of the *Arabidopsis* chromosomes. The availability of the uni-cDNA collection will eliminate the need for cDNA library construction and will allow the creation of a library where every cDNA will be represented at equimolar concentration. More importantly, the full-length cDNA collection will allow the biochemical characterization of the *Arabidopsis* proteome. Concomitantly, the available DNA sequence and chip technology eliminate the need for Southern and northern analyses in *Arabidopsis*. Isolation of insertional mutants for all the genes on the chromosomes will offer additional resources to elucidate the function of the genes by reverse genetics. The biological resources that will be available for *Arabidopsis* will allow plant biologists to advance plant science and agriculture to levels never dreamed of 30 years ago. □

Methods

We used two BAC and one YAC libraries made from the ecotype Columbia of *Arabidopsis thaliana*: the TAMU¹³ BAC library (T); the IGF¹⁴ BAC library (F); and the yUP²⁸ YAC library. BAC DNA was hydrodynamically sheared²⁹ to 1–2 kb and the DNA was ligated into an M13 vector using a 'linker-adapter system'³⁰ that minimizes the formation of chimeric DNAs. We used an automated sample preparation system for template preparation³¹. We sequenced each BAC to 10× coverage using Dyeprimer chemistry. Individual BACs were assembled from the shotgun sequences using Phred^{32,33}/Phrap (<http://www.phrap.org/>)

Consed³⁴, and the gaps in each BAC were closed using a combination of BAC walking, directed PCR and resequencing of individual clones using Dye terminator chemistry. All sequences are >97% double-stranded and have no more than one error per 25 kb. BACs were sequenced at TIGR as described³.

We analysed the DNA sequence of each BAC for protein-coding genes using gene-prediction software and alignments with sequences from the EST and non-redundant protein databases. For initial gene and exon predictions, we used GRAIL³⁵, Genscan³⁶, fexa (V. Solovyev, <http://genomic.sanger.ac.uk/gf/gf.shtml>), GlimmerA³⁷ and GeneMark.HMM³⁸. Splice site predictions were made using GeneSplicer (M. Pertea and S. Salzberg, unpublished software) and NetPlantGene³⁹. We aligned sequences in the EST and non-redundant protein databases to the BAC sequences using the BLAST⁴⁰ and AAT⁴⁰ sequence database search and alignment software. Protein gene models were then assigned both automatically by the PEDANT⁴¹ system and manually by evaluating and editing the gene prediction and database alignment data for each BAC. We identified tRNA genes using the program tRNA-scan-SE⁴². We identified potential transit and targeting peptide sequences in proteins using TargetP⁴³ (specificity >0.95), and repeats using Repeat Masker (A. F. Smit & P. Green; <http://ftp.genome.washington.edu/RM/RepeatMasker.html>; <http://www.girinst.org>).

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Supplementary Information is available at Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Correspondence and requests for materials should be addressed to A.T. (e-mail: theo@nature.berkeley.edu) or J.R.E. (e-mail: ecker@salk.edu). The accession members for chromosome 1 are: northern arm, AE005172; southern arm AE005173.

Sequence and analysis of chromosome 3 of the plant *Arabidopsis thaliana*

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Arabidopsis thaliana is an important model system for plant biologists¹. In 1996 an international collaboration (the *Arabidopsis* Genome Initiative) was formed to sequence the whole genome of *Arabidopsis*² and in 1999 the sequence of the first two chromosomes was reported^{3,4}. The sequence of the last three chromosomes and an analysis of the whole genome are reported in this issue^{5–7}. Here we present the sequence of chromosome 3, organized into four sequence segments (contigs). The two largest (13.5 and 9.2 Mb) correspond to the top (long) and the bottom (short) arms of chromosome 3, and the two small contigs are located in the genetically defined centromere⁸. This chromosome encodes 5,220 of the roughly 25,500 predicted protein-coding genes in the genome. About 20% of the predicted proteins have significant homology to proteins in eukaryotic genomes for which the complete sequence is available, pointing to important conserved cellular functions among eukaryotes.

Chromosome 3 is submetacentric and represents about 20% of the *Arabidopsis* genome. It has been estimated, using yeast artificial chromosome (YAC)-based physical maps^{9,10}, to be 21–23 Mb long (excluding the centromeric and telomeric regions). We sequenced 330 clones (bacterial artificial chromosomes (BACs)^{11,12}, P1 clones¹³ and transformation-competent artificial chromosomes (TACs)¹⁴) and eight polymerase chain reaction (PCR) products and assembled them into four contigs representing 23,172,617 base pairs (bp) of non-redundant sequence. The bottom arm contains a residual sequencing gap of around 5 kilobases (kb). Of the approximately 150 and 450 kb of sequence in the two small centromeric contigs,

340 kb (90 and 250 kb, respectively) correspond to high accuracy DNA sequence; the rest consists of unfinished highly repetitive BAC sequences.

For each chromosome arm, the canonical telomeric repeats¹⁵ specific for chromosome ends border a long euchromatic region (~11 and ~7 megabases (Mb) for the top and bottom arms, respectively) characterized by a high and roughly uniform gene density. The gene density then gradually decreases as the retro-transposon density increases towards the peri-centromeric/centromeric heterochromatic region. The top arm contig terminates at the F15D2 BAC clone, which contains at its end a 180-bp tandem repeat characteristic of the *Arabidopsis* centromere^{16,17}. The bottom arm contig begins at the F4M19 BAC clone with a 5S ribosomal DNA (rDNA) repeat cluster¹⁸. The two small centromeric contigs were mapped in between the two arm contigs by tetrad analysis⁸. The relative positions and orientations of these small contigs have not yet been confirmed experimentally. The probable structure of the chromosome 3 centromere is shown in ref. 7 and in Supplementary Information. The size of the genetically defined centromeric region is estimated to be around 1.7 Mb; added to the size of the chromosome arms, this indicates a size of 24 Mb for the whole chromosome. Unexpectedly, the centromeric region contains, in addition to known repetitive elements⁷, a block of 40 nearly perfect telomeric repeat units and a single complete rDNA unit (over 99% identical in the 25S, 18S and 5.8S regions). A general description of the characteristics of

Table 1 Features of chromosome 3

(a) The DNA molecule	
Length	23,172,617 bp
Top arm	13,590,268 bp
Bottom arm*	9,582,349 bp
Base composition (%GC)	
Overall	35.4
Coding	44.3
Non-coding	33.0
Number of genes	5,220
Gene density	4.5 kb per gene
Average gene length	1,925 bp
Average peptide length	424 amino acids
Exons	
Number	26,570
Total length	6,654,507 bp
Average per gene	5.1
Average size	250 bp
Introns	
Number	21,350
Total length	3,397,531 bp
Average size	159 bp
Percentage of genes with ESTs†	59.8%
Number of ESTs‡	20,732
(b) The proteome	
Total proteins	5,220
Proteins with INTERPRO domains	2,989 (57.8%)
Genes which contain at least one transmembrane domain	1,615 (30.9%)
Genes which contain at least one SCOP domain	1,664 (31.9%)
Secretory pathway default value‡	877
Secretory pathway >0.95 specificity	813
Chloroplast default value	754
Chloroplast >0.95 specificity	420
Mitochondria default value	554
Mitochondria >0.95 specificity	63
Functional classification	
Cellular metabolism	745
Transcription	566
Plant defence	354
Signalling	356
Growth	357
Protein fate	314
Intracellular transport	269
Transport	155
Protein synthesis	148
Total	3,264

* The size of the bottom arm included the two small centromeric contigs (~340 kb).

† EST matches were calculated using a similarity threshold of 90%.

‡ The assignment to secretory pathway, chloroplast and mitochondria result from a TargetP analysis.

chromosome 3 is available as Supplementary Information.

To annotate chromosome 3 we combined *in silico* gene-finding methods and sequence comparisons with external databases, followed by human inspection. Chromosome 3 contains 5,220 putative genes. A 1.9-kb-long gene (from start to stop codon) is predicted on average every 4.5 kb and contains from 1 (21%) to 78 exons; therefore, 43% of the DNA potentially encodes proteins. The characteristics of the genes predicted on this chromosome are essentially similar to those described for chromosomes 2 (ref. 4) and 4 (ref. 3), and are shown in Table 1(a). To assign unambiguously *Arabidopsis* expressed sequence tags (ESTs) to their cognate genes, a whole-genome comparison was performed and for each EST the best match with the predicted gene was retained. We know that at least 2,714 (53%) of the predicted genes are expressed, because there are corresponding complementary DNAs or ESTs. Of the 39 genes represented by more than 50 ESTs, 18% are located in a 165-kb interval, mainly owing to a cluster of putative lectin genes that has nine members and accounts for 349 ESTs. Alternative splicing has been reported in *Arabidopsis* genes^{19–21}. We detected potential alternative splicing for 1–2% of around 1,000 predicted genes with at least three EST and/or messenger RNA matches, which confirms that alternative splicing is rare in chromosome 3 compared with its frequency in mammalian genes²², at least in the set of ESTs available.

We found two types of duplication, represented either by clustered gene families or by segmental duplications. Eight hundred and thirty seven predicted genes and 47 predicted pseudogenes are members of 306 clustered gene families with between 2 and 23 members each. The percentage of the predicted genes showing a match with an EST is 38%, which is significantly ($P < 10^{-3}$) lower than the percentage of matches between ESTs and the overall predicted genes on chromosome 3 (52%). The low expression of these genes suggests that some of them may be vestigial. Other possibilities such as gene silencing or the acquisition of specific expression patterns could also explain this feature. In most cases it is likely that clustering is due to simple amplification by duplication. When considering only clustered gene families with two members, 86% of the gene pairs are on the same strand. Remnants of very recent duplications (for example, AT3g24870 and AT3g24880) which show over 98% identity (including introns) have been identified. In some cases, the organization of the cluster is much more complex and its evolution is more difficult to trace (for example, AT3g59150 to AT3g59270). In addition to the clustered gene families, there are large segmental duplications between the chromosome 3 sequence and sequences on other chromosomes, such as a 4-Mb duplication between chromosomes 3 and 2 (ref. 7). The analysis of the 45 chromosome 3 clustered gene families in this

duplication shows that, in about 80% of cases, the best match is found in the same cluster on chromosome 3, indicating that most of these clustered gene families were probably created after the segmental duplication.

The characteristics of the 5,220 putative proteins encoded by this chromosome are shown in Table 1(b). Four hundred and twenty seven (8.3%) were already known at least by the presence of an mRNA in the sequence databases. For about 60% of the annotated proteins, we can predict a putative function using sequence similarity criteria and INTERPRO²³ analysis. Results from these two programs do not completely overlap, which further increases the percentage for which a putative function can be assigned. The remaining group consists of proteins predicted by gene-finding programs only and of proteins with similarity to other proteins of unknown function. The distribution of the functional categories is in good agreement with those reported for the *Arabidopsis* genome as a whole⁷.

Some gene functions are over-represented in the 5,220 genes predicted on chromosome 3, in part owing to the presence of clustered gene families. Of the 20 *Skp1* homologues found in the *Arabidopsis* genome, eight are on chromosome 3, and six of these are found in two clustered gene families. In yeast *Skp1* is involved in specific protein degradation via the Skp1-Cdc53-F-box pathway, and a similar role has been proposed in *Arabidopsis*²⁴. Three of the four nitrilase genes in the *Arabidopsis* genome were found on chromosome 3 as a clustered gene family.

Chromosome 3 harbours some unexpected features, such as a roughly 5-kb chloroplast DNA insertion, the complete rDNA repeat unit and the telomeric repeat, all in the genetically defined centromere. Although the biological analysis of the chromosome 3 genes has to be placed in the context of the whole genome⁷, chromosome 3 contains a number of homologues to human disease genes (Table 2) which point to important conserved cellular functions in eukaryotes. Most if not all are involved in basic cellular mechanisms. The analysis of homologues of human genes can provide some clues about the function of these genes in a plant. Chromosome 3 contains a putative orthologue of the human *DEK* gene, which is involved in acute myelogenous leukaemia. This gene induces alterations of the superhelical density of DNA in chromatin²⁵. We also detected homologous genes to both human *SKI2W* and yeast *Ski2* (ref. 26). The yeast gene regulates expression of non-poly-A mRNA and has antiviral activities, and it is tempting to attribute an antiviral defence role to this gene in *Arabidopsis*. Inter-genome comparisons will be of great interest for accelerating the understanding of gene function. In this respect *Arabidopsis* will be extremely useful in providing information on genes common to different organisms, owing to the relative ease of screening for mutants in a selected gene. Most of the general features are consistent with those reported for the other chromosomes. For example, the gene density is similar for all the *Arabidopsis* chromosomes. This highly conserved gene density can be explained by both the very high number of segmental duplications⁷ and a continuously very high rate of reshuffling during the evolution of the *Arabidopsis* genome. □

Methods

We used two strategies to establish the sequence of chromosome 3. The first was based on the construction of a fine physical map by ordering P1, TAC and BAC clones using DNA markers and clone end sequences²⁷. Under the second strategy we isolated seeded clones using STS-selected markers and sequenced them by shotgun sequencing. Seeded clones were then extended in both directions by searching for sequence identities in the BAC end sequence database, which was then cross-examined with BAC fingerprint data. When necessary PCR products were sequenced to fill gaps between two contigs. Individual BAC clones were assembled from the shotgun sequences. Gaps between contigs were closed by primer walking and low-quality regions were finished by resequencing. Individual BAC clone assemblies were checked by restriction digests and the error rate of the final sequence was estimated to be lower than $1 \text{ in } 3 \times 10^5$ by comparing independent sequence overlaps representing ~950 kb. Of the 27 discrepancies found, 10 were sequencing errors and the remainder are probably due to BAC mutations.

The annotation of the sequences was performed as described^{3,4,28}. Alignments were computed using a SMITH-WATERMAN²⁹ algorithm implemented in LASSAP³⁰ (large scale sequence comparison package) version 1.2.0a.

Table 2 Homologues of human genes with best matches on chromosome 3

Human gene	Identity*	Possible function	<i>Arabidopsis</i> gene
Acute myeloid leukaemia (DEK)	36% (138 aa)	Chromatin structure	AT3g48710
Ataxia telangiectasia (ATM)	38% (1,042 aa)	DNA repair	AT3g48190
Retinoblastoma (RB1)	23% (850 aa)	Regulation of apoptotic function	AT3g12280
Machado-Joseph (MJD1)	36% (244 aa)	Protein folding	AT3g54130
Miller-Dieker lissen. (PAF)	32% (312 aa)	Phospholipase	AT3g49660
Myotubular myopathy 1 (MTM1)	35% (454 aa)	Signalling pathway	AT3g10550
Coffin-Lowry (RPS6KA3)	47% (323 aa)	Ribosomal protein S6 kinase	AT3g08720
Williams-Beuren (ELN)	31% (762 aa)	Composition of elastic fibres	AT3g22140
Carnitine deficiency (SLC22A5)	31% (453 aa)	Sodium carnitine cotransporter	AT3g20660
Downregulated in adenoma (DRA)	27% (506 aa)	Anion transport	AT3g12520

* Amino-acid identity over the homologous region of the gene; the size of the region is given in parentheses.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Correspondence and requests for materials should be addressed to M. Salanoubat (e-mail: salanoubat@genoscope.cns.fr). The annotated set of chromosome 3 genes is available at <http://www.kazusa.or.jp/kaos/>, <http://www.mips.biochem.mpg.de/> and at <http://www.tigr.org>. All individual BACs have been submitted to EMBL, GB and DDBJ.

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Sequence and analysis of chromosome 5 of the plant *Arabidopsis thaliana*

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The genome of the model plant *Arabidopsis thaliana* has been sequenced by an international collaboration, The Arabidopsis Genome Initiative. Here we report the complete sequence of chromosome 5. This chromosome is 26 megabases long; it is the second largest *Arabidopsis* chromosome and represents 21% of the sequenced regions of the genome. The sequence of chromosomes 2 and 4 have been reported previously^{1,2} and that of chromosomes 1 and 3, together with an analysis of the complete genome sequence, are reported in this issue³⁻⁵. Analysis of the sequence of chromosome 5 yields further insights into centromere structure and the sequence determinants of heterochromatin condensation. The 5,874 genes encoded on chromosome 5 reveal several new functions in plants, and the patterns of gene organization provide insights into the mechanisms and extent of genome evolution in plants.

We determined the sequence of chromosome 5 from 403 overlapping bacterial artificial chromosome (BAC), phage (P1) and transformation-competent artificial chromosome (TAC) clones⁶⁻⁸, comprising two contigs representing the chromosome arms. The order of clones was selected using fingerprint contigs, BAC end sequencing and Southern blotting as described previously^{2,9}, starting from a set of clones anchored to the genetic map by marker content. Regions of sequence have been published previously as part of the sequence release policy of The Arabidopsis Genome

Initiative^{10,11}. At present, three centromeric BACs and roughly 7 kilobases (kb) in two regions of significant sequence complexity are still being sequenced.

We assigned 30 sequenced markers unambiguously to chromosome 5 and their order was confirmed on a recombinant inbred map¹². We sequenced telomeric regions and integrated them with genome sequence using genomic DNA in polymerase chain reactions (gPCR). The sequence of heterochromatic regions flanking the centromere was determined up to and including regions of 180-base-pair (bp) centromeric repeats. The features of chromosome 5 are summarized in Table 1; for a graphical image of chromosome 5 displaying genes, repeats and other features, see Supplementary Information Fig. 1. The total sequenced regions comprise 25,953,409 bp; together with roughly 250 kb of 5S repeat sequence and around 1,000 kb of unsequenced 180-bp repeats^{9,13}, this yields an estimated chromosome size of 27.2 megabases (Mb). This is less than the 28.4 Mb predicted by physical mapping⁹; the difference is probably due to overlaps of unknown size between clones. The average gene density on chromosome 5 is close to that described for the entire genome⁵, with about 1 gene every 4.4 kb (Table 1(a)). Gene density varies along the chromosome, with a lower average gene density close to the centromeric region, similar to that reported for chromosomes 2 and 4 (ref. 14).

The sequence extends from centromeric repeats into telomeric regions, resembling that achieved for the arms of other chromosomes¹⁻⁴. Subtelomeric repeats, characterized by short direct repeats and degenerate telomeric sequences, separated regions of normal gene density from the telomeric repeats (T₃AG₃) proper. Yeast artificial chromosome (YAC) and BAC contigs covering the centromeric region^{9,13} have been constructed, and the repeat content and distribution have been analysed in detail¹⁴. The interspersed pattern of 180-bp repeat clusters was similar to that on the centromere of chromosome 4 (CEN4; ref. 2). We found two clusters of 5S ribosomal DNA flanking the central 180-bp repeat domain in the sequence, confirming the two 5S loci near CEN5 revealed by fluorescence *in situ* hybridization (FISH, Fig. 1) in several ecotypes¹⁵. The short 5S cluster on the long arm is entirely contained

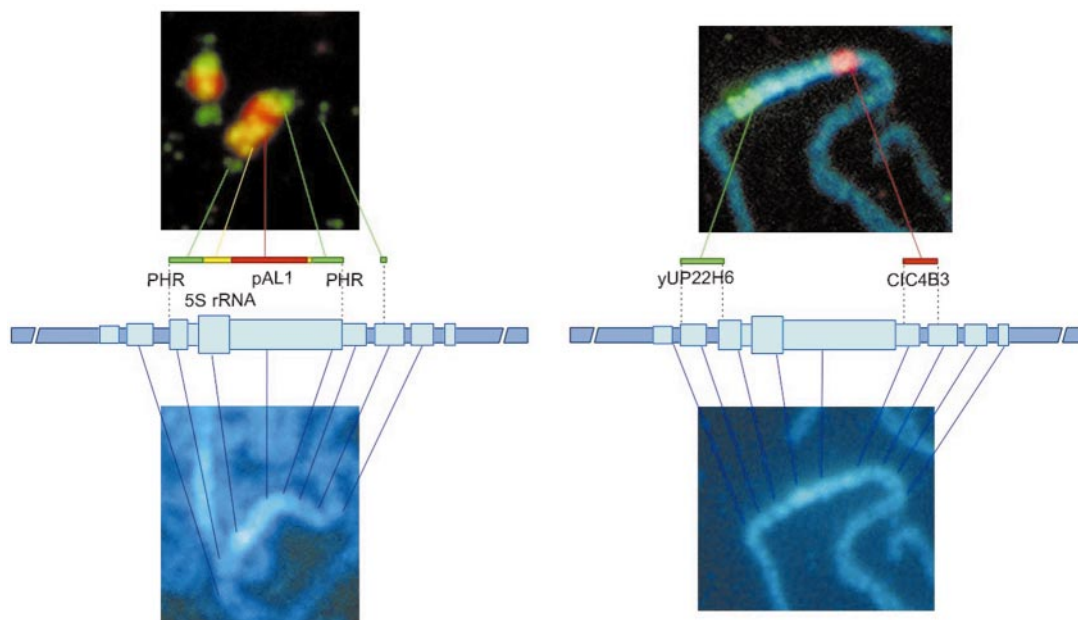


Figure 1 FISH analysis of heterochromatin showing CEN5 features. The positions of 5S rDNA (yellow), pAL1 180-bp repeats (red) and pericentromeric Athila retroelements (green) are shown in the left panel. These features are linked to the physical map using YACs yUP22H6 (green) and CIC4B3 (red) to define the short and long arm, respectively (right

panel). DAPI-stained pachytene cells reveal several knobs with variable intensity near CEN5. The heterochromatic knob hk5L is visible as a small island of green hybridization signals (left panel).

within BAC T25B21 and the long cluster is flanked by BACs T26N4 and T3P1. Contiguous sequence extends from both 5S clusters into the flanking regions of the central 180-bp domain bounded by BACs T21M13 and F13C19.

Sequence from the central heterochromatic domain is characterized by a relatively low gene density, increased repeat density (including the centromere-associated *Athila* retroelement) and increased pseudogene density (see Supplementary Information Fig. 1). Expressed genes in this central region of CEN5 include those encoding galactinol synthase and a phosphate/phosphoenolpyruvate translocator precursor. More extensive heterochromatin flanks the 5S clusters, as shown in Fig. 1. Within these heterochromatic tracts, gene density varies substantially, unlike the gradual reduction in gene density observed in centromere-proximal regions of chromosomes 2 and 4. This patchy distribution of heterochromatin can be seen in images stained with 4,6-diamidino-2-phenylindole (DAPI; Fig. 1), consistent with a proposed chromomere (differentially stained chromosomal region) model²⁹.

On the long arm, retroelements are found in patches that correlate with heterochromatin features. FISH analysis with a BAC probe containing *Athila* retroelements revealed a heterochromatic knob, named hk5L, in the region of YAC C1C5B3, which underlies the sequence containing the repeats (Fig. 2). There were eight DNA transposons and eighteen retrotransposons in the region corresponding to hk5L. We found 30 tandem repeats of a 2,200-bp sequence, with an insertion of *En-Spm*-like transposon. This 2,200-bp element had no significant similarity to the 1,950-bp tandem repeat element in the heterochromatic knob hk4S on chromosome 4 (refs 16, 17) and did not include the terminal direct repeat structure that was found in the 1,950-bp element. Tandem repeats are generally associated with heterochromatic domains, such as the nucleolar organizers, heterochromatic knobs and pericentromeric heterochromatin^{16–18}. This structural conservation indicates the importance of tandem repeat clusters in heterochromatin formation, and suggests that this sequence organization, rather than sequence content, is important in chromosome condensation.

Chromosome 5 contains 4,110 genes encoding proteins of predicted function. Proteins involved in metabolism (21.1%), transcription (18.6%) and defence (11.9%) comprise the major classes (Table 1(b)), and these proportions are consistent with similar analysis of the whole genome⁵. Chromosome 5 contains 37 families of genes encoding proteins of predicted function, present as tandem arrays of more than three members, consistent with the high proportion of gene families observed in the other chromosomes. The largest cluster contains an array consisting of five germin (oxalate oxidase) genes, four receptor-like kinase genes, a single transporter gene and three acyl transferase genes (one of which is interrupted by a retroelement), followed by another nine germin genes.

A number of genes on chromosome 5 exhibit a high degree of overall similarity to genes of known function in other organisms that have not been previously identified in *Arabidopsis*. These include the gene for the minD septum-site determining protein, which is highly conserved in *Chlorella*, *Synechocystis* and *Escherichia coli*. It is required, together with the minC and MinE proteins, for proper placement of the septum in cell division¹⁹. It may be involved in organelle replication, although it is not reliably predicted to possess a transit peptide. A protein very similar to the product of the *Drosophila* separation anxiety gene encodes a possible *N*-acetyl transferase. Mutation of the *Drosophila* and human genes impairs chromosome spindle assembly and formation, leading to non-disjunction at first meiosis, which causes behavioural differences in flies and humans. Chromosome 5 encodes a homologue of Notchless²⁰, which modifies Notch activity in cell signalling that determines cell fate in *Drosophila*. This suggests related functions in signal transduction in plants, although proteins related to Notch are not found in *Arabidopsis*⁵.

Eighty-eight genes on chromosome 5 have high overall similarities ($E < 10^{-15}$) to the 289 genes involved in human disease syndromes established for comparison with the *Drosophila* genome²¹. Most of these are also highly conserved between *Drosophila* and *Caenorhabditis elegans*, revealing a significant potential for *Arabidopsis* biology to contribute to our knowledge of human disease conditions. Several of these genes belong to classes

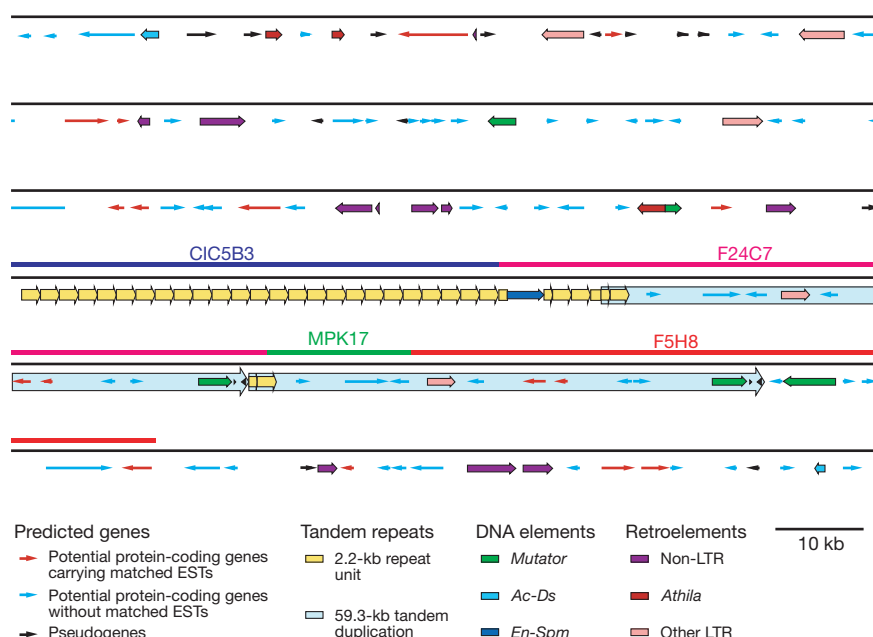


Figure 2 Sequence features of the heterochromatic knob region. The diagram shows a 0.6-Mb region surrounding the heterochromatic knob. Predicted protein coding genes are

shown as thin arrows. Tandem repeats and transposons are indicated by colour-coded block arrows.

Table 1 Features of chromosome 5

(a) The DNA molecule	
Length	25,953,409 bp
Top arm	11,132,192 bp
Bottom arm	14,803,217 bp
Base composition (%GC)	
Overall	34.5
Coding	44.1
Non-coding	32.5
Number of genes	5,874
Gene density	4.4 kb per gene
Average gene length	1,974 bp
Average peptide length	429 amino acids
Exons	
Number	31,226
Total length	7,571,013 bp
Average per gene	5.3
Average size	242 bp
Introns	
Number	25,352
Total length	4,030,045 bp
Average size	159 bp
Percentage of genes with ESTs	61.4%
Number of ESTs	22,885
(b) The proteome	
Total proteins	5,874
With INTERPRO domains	3,136 (53.4%)
Proteins containing ≥ 1 transmembrane domain	1,940 (33.0%)
Proteins containing ≥ 1 SCOP domain	2,121 (36.1%)
Secretory pathway (default value)	1,014 (17.3%)
Secretory pathway >0.95 specificity	964 (16.4%)
Chloroplast (default value)	887 (15.1%)
Chloroplast >0.95 specificity	475 (8.1%)
Mitochondria (default value)	627 (10.7%)
Mitochondria >0.95 specificity	65 (1.1%)
Functional classification	
Cellular metabolism	868 (21.1%)
Transcription	763 (18.6%)
Plant defence	490 (11.9%)
Signalling	420 (10.2%)
Growth	469 (11.4%)
Protein fate	395 (9.6%)
Intracellular transport	334 (8.1%)
Transport	206 (5.0%)
Protein synthesis	165 (4.0%)
Total	4,110

encoding proteins of deeply conserved function, such as DNA excision repair genes (implicated in xeroderma pigmentosum), a retinis pigmentosa RPGR gene homologue encoding a characterized *Arabidopsis* UVB resistance gene, and ATP-dependent copper transporters (implicated in Wilson's and Menke's diseases). In the latter case the *Arabidopsis* homologues are more similar to the human counterpart than to the *Drosophila*, *C. elegans* or yeast homologues.

The 5,874 predicted genes on chromosome 5 include many genes with significant similarity to genes from other organisms, including human. This indicates the potential utility of *Arabidopsis* sequence in applications beyond crop plant improvement. The large-scale application of functional genomics tools is required for the systematic identification of the cellular roles of 1,764 predicted genes encoding proteins of no known function. The community of *Arabidopsis* researchers possesses the organization and techniques²² to accomplish this task efficiently. □

Methods

We used a clone-based strategy to assemble sequence from the Columbia ecotype as described^{2,9}. We assessed sequence accuracy by comparison of overlapping sequence of adjacent BACs, and 15 mismatches were found and corrected. A 233-kb region was sequenced by two laboratories independently to assess accuracy and consistency of assembly, and we found no differences. We analysed sequence assemblies for frame shifts and corrected them if necessary. The DNA sequence was analysed as described² except that the gene-prediction tools Genemark.hmm²³ and GENSCAN-2 (ref. 24) were used. We integrated the output of a combination of gene-prediction programs with predicted exon-intron structures²³ to derive polypeptide sequences. Similarities between the

encoded polypeptides and other proteins were established and classified using BLAST²⁶ and INTERPRO²⁷ and structural motifs classified using PEDANT²⁸. We determined the predicted functions of genes by significant homology with genes of known function from other organisms.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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<http://www.kazusa.or.jp/kaos/>, <http://www.mips.biochem.mpg.de/proj/thal/> and <http://www.tigr.org/tdb/ath1/htmls/ath1.html>.

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Geomagnetic intensity variations over the past 780 kyr obtained from near-seafloor magnetic anomalies

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Knowledge of past variations in the intensity of the Earth's magnetic field provides an important constraint on models of the geodynamo. A record of absolute palaeointensity for the past 50 kyr has been compiled from archaeomagnetic and volcanic materials, and relative palaeointensities over the past 800 kyr have been obtained from sedimentary sequences. But a long-term record of geomagnetic intensity should also be carried by the thermoremanence of the oceanic crust. Here we show that near-seafloor magnetic anomalies recorded over the southern East Pacific Rise are well correlated with independent estimates of geomagnetic intensity during the past 780 kyr. Moreover, the pattern of absolute palaeointensity of seafloor glass samples from the same area agrees with the well-documented dipole intensity pattern for the past 50 kyr. A comparison of palaeointensities derived from seafloor glass samples with global intensity variations thus allows us to estimate the ages of surficial lava flows in this region. The record of geomagnetic intensity preserved in the oceanic crust should provide a higher-time-resolution record of crustal accretion processes at mid-ocean ridges than has previously been obtainable.

Past geomagnetic intensity fluctuations provide important constraints for geodynamo models¹. Absolute palaeointensities from archaeomagnetic and volcanic materials are sufficiently abundant to document dipole intensity changes since about 50 kyr ago². However, the scarcity of such data from earlier periods allows only the most general features of the intensity record to be determined³. An increasing number of relative palaeointensity records are available from sedimentary sequences, particularly from the Brunhes⁴. However, uncertainties remain concerning the remanence acquisition process in sediments and the suitability of normalizing parameters required to estimate relative palaeointensity⁵. Furthermore, the calibration of these relative intensity records against the best-documented (Holocene) portion of the absolute intensity record is generally not possible because the uppermost sediments are often missing or disturbed.

Marine magnetic anomalies provide a third method for determining past geomagnetic intensity fluctuations. Coherent short wavelength variations in sea surface magnetic anomalies may be of geomagnetic origin, recording either short polarity reversals⁶ or palaeointensity variations^{7,8}. Palaeointensity variations have also been suggested as the cause of even shorter wavelength features observed in near-bottom magnetic anomaly data^{9,10}. However, the role of geomagnetic intensity in modulating anomaly amplitudes remains controversial.

Geomagnetic intensity variations in the Brunhes epoch

To assess the presence of a geomagnetic intensity signal within the ocean crust, we collected near-bottom magnetic anomaly data across the spreading-axis central anomaly near 19.7° S, 19.4° S and 17.3° S on the East Pacific Rise (EPR). The presence of large seamounts in the 17.3° S survey area¹¹ limited these profiles to the younger portion of the Brunhes. In contrast, the EPR 19–20° S shows a remarkably simple (albeit asymmetric) spreading history throughout the Brunhes, with no seamount chains or evidence of significant axial discontinuities¹¹. We focus here on eight profiles from the faster spreading east flank at 19–20° S that span the entire central anomaly (Fig. 1a). This area is ideal for evaluating the geomagnetic contribution to anomaly variations within the

Brunhes because the extremely fast spreading rate ($\sim 142 \text{ mm yr}^{-1}$ full spreading rate; 76 mm yr^{-1} east flank) provides the maximum possible temporal resolution. Perhaps most importantly, independent information on field fluctuations over the past 800 kyr⁴ are available for direct comparison with the anomaly data.

Both sea surface and near-bottom magnetic anomaly data at 19–20° S show several highly correlated short wavelength features within the Brunhes. As a first-order measure of coherent anomaly variations that may represent geomagnetic intensity variations, we have calculated simple stacks (averages) of the anomaly profiles (Fig. 1b and c). Individual profiles were deskewed¹² and stretched to a common length, using as tie points only the location of the ridge axis and the Brunhes/Matuyama boundary (0.78 Myr ago; ref. 13), which varies by about 1 km among the eight profiles. Comparison of the stacked near-bottom anomaly profile with the sedimentary relative palaeointensity record (Fig. 1e) of ref. 4 reveals a number of similar features (such as lows at ~ 200 , 380 and 700 kyr ago). Ages of several distinctive near-bottom magnetic anomalies may be inferred by comparison with the isotopically dated sedimentary palaeointensity record. These near-bottom anomalies, as well as the corresponding lower-resolution sea surface anomalies, should provide additional temporal markers within the Brunhes.

Because the anomaly data were measured along uneven tracks at a variable distance above the sea floor, we have also inverted the magnetic anomaly data to provide an estimate of the magnetization variations. The resulting inversion solution stack (Fig. 1d) closely resembles the sedimentary palaeointensity record. It retains nearly all the features present in the original stacked anomaly data, including even subtle variations such as the double anomaly/magnetization peak centred at 420 kyr ago. We suggest that additional factors that affect the anomaly amplitudes (geochemistry, alteration, layer thickness) are unlikely to be coherent along-strike over the distance (60 km) spanned by our anomaly profiles or to fortuitously parallel the sedimentary palaeointensity curve. We conclude that fluctuations in geomagnetic field intensity are the dominant cause of the coherent magnetization variations within the Brunhes-age crust in this area.

Near-ridge anomalies and absolute palaeointensities

A further test of the importance of geomagnetic intensity in controlling crustal magnetization is possible in the near-ridge environment, over timescales (0–50 kyr ago) where the independent estimates of field intensity are best constrained. Absolute palaeointensity determinations from archaeomagnetic and volcanic materials² document a substantial dipole intensity increase from a low of $\sim 2 \times 10^{22}$ A m² at ~ 45 kyr ago to a peak value of $\sim 11 \times 10^{22}$ A m² at 1–3 kyr ago, followed by a rapid decrease to the present value of 7.91×10^{22} A m² (Fig. 2a). The stacked sedimentary palaeoin-

tensity record of ref. 4, calibrated to absolute intensities over the interval 10–40 kyr ago, can be used to extend the palaeointensity record to older ages. Together these data give a record of dipole field fluctuations within the youngest portion of the Brunhes that may be compared to palaeointensity and anomaly data from the EPR. If geomagnetic intensity is the dominant factor determining crustal magnetization intensity, we expect the near-bottom anomaly data and inversions to show moderate values at the axis, flanked by higher values 1–2 km off-axis and much lower values over crust generated during the low field intensities at about 45 kyr ago.

The stack of near-bottom magnetic anomaly profiles (Fig. 1c) shows precisely the expected pattern, with a short wavelength minimum superimposed on the pronounced axial anomaly high (termed the central anomaly magnetization high, CAMH¹⁴). This

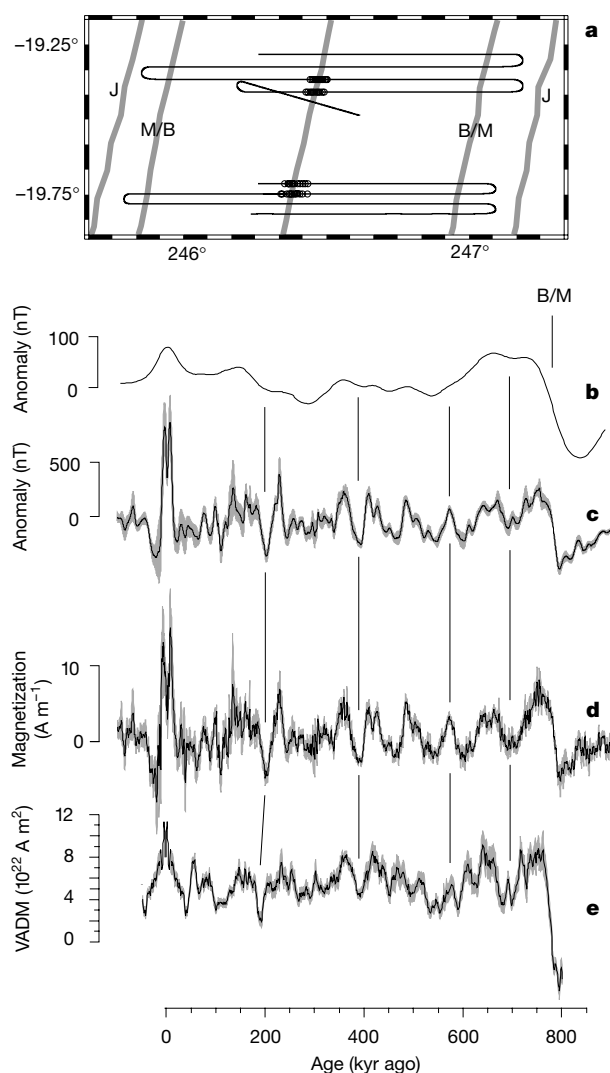


Figure 1 Location of survey and comparison of geomagnetic intensity variations in Brunhes from sedimentary records and near-bottom magnetic anomalies. **a**, Location of near-bottom and sea surface profiles. Open circles indicate location of glass samples. Location of Brunhes/Matuyama (B/M) and Jaramillo (J) shown for reference. **b**, Stack of sea surface anomaly profiles coincident with near-bottom survey lines. Near-bottom magnetic anomaly (**c**) and inversion solution (**d**) stacks incorporate eight profiles from the faster-spreading east flank of the East Pacific Rise (EPR) 19–20° S. Inversion solutions are for constant source thickness (0.5 km), calculated using a direct inversion method for an uneven track and source topography (R. L. Parker, personal communication). Because longer wavelengths in the inversion solutions are poorly constrained, magnetization variations with wavelengths greater than 10 km have been removed before stacking. Ages calculated assuming constant spreading and age of 0.78 Myr for Brunhes/Matuyama (B/M) boundary¹³. **e**, Sedimentary relative palaeointensity stack⁴ (10–800 kyr ago) combined with global archaeomagnetic data² (0–10 kyr ago). Younger portion of record has been mirrored for comparison with seafloor records. VADM, virtual axial dipole moment. Standard errors (grey) shown for all three records.

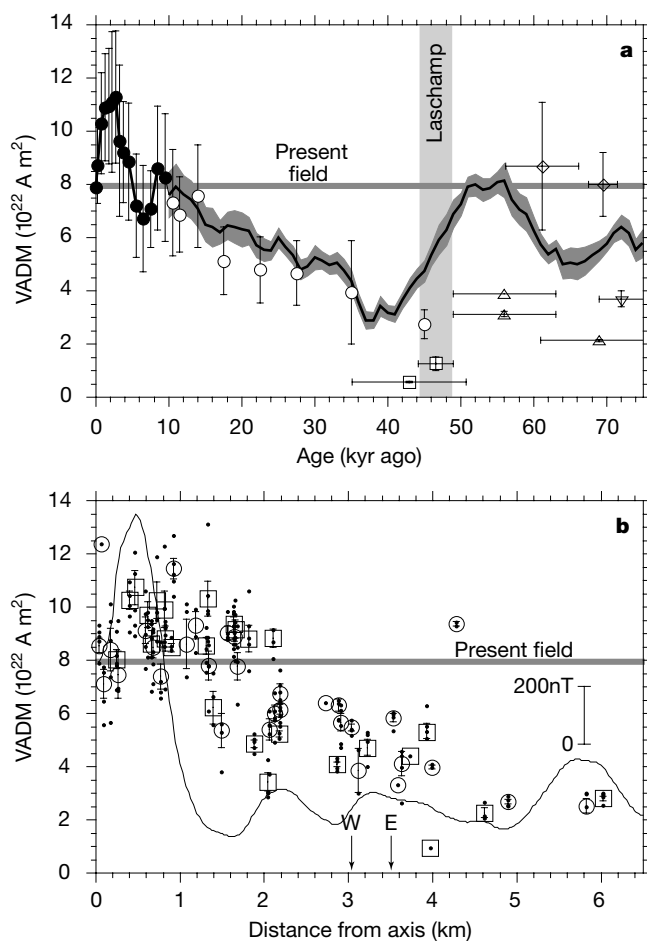


Figure 2 Comparison of absolute palaeointensities from seafloor glass samples with independent estimates of geomagnetic field intensity. **a**, Line with associated standard error (grey band) is relative intensity variation from stacked sedimentary records⁴, calibrated to absolute intensities from volcanic materials (open circles). We use the calibrated sediment record (>10 kyr ago), together with archaeomagnetic results (<10 kyr ago; filled circles) as the best record of intensity fluctuations (heavy line) in subsequent modelling. Archaeomagnetic and volcanic data from compilation of ref. 2. Palaeointensities from Laschamp excursion lavas (squares) in Iceland and France^{24,25}, although not included in compilation², may suggest even lower dipole intensity near 45 kyr ago. More recent palaeointensities from samples with radiometric dates in the interval 50–75 kyr ago shown by diamonds³⁶, triangles¹⁸, inverted triangles³⁷. Grey bar indicates best estimate for the age of the Laschamp excursion. **b**, Virtual axial dipole moments (VADM) for individual glass specimens (dots) and weighted site means with standard errors¹⁹. Circles (squares) indicate site means for faster-spreading east flank (slower west flank). Arrows indicate the nominal position of the Laschamp excursion based on simple constant spreading. Stacked anomaly record is shown for comparison.

pattern is particularly evident in individual profiles near 19.7° S (see Supplementary Information), where the CAMH has a pronounced notch and flanking highs that reach amplitudes of more than 3,000 nT. The axial anomaly is similar, though less symmetric and lower amplitude, in profiles near 19.4° S. This pattern is also evident in the inversion solution stack derived from the profiles at 19–20° S (Fig. 1d). In contrast, the CAMH in the 17.3° S study area is more complex (presumably reflecting the pattern of crustal accretion) and has markedly lower amplitude (~500–750 nT) which we attribute primarily to the lower iron content of the lavas¹⁵.

As a more direct test of the importance of geomagnetic intensity in controlling crustal magnetizations, we determined absolute palaeointensities on 228 basaltic glass chips recovered by wax coring at sites along selected near-bottom profiles (see Supplementary Information). Submarine basaltic glass is nearly ideal for palaeointensity studies, with the remanence residing predominantly in single-domain magnetite grains that alter little during laboratory heating¹⁶. Glass samples from recent flows on both the EPR and the Juan de Fuca ridge have recovered the geomagnetic field intensity at these respective sites^{17,18}.

Using the modified Thellier double heating method¹⁹, we find glass palaeointensities that range from about 50% above the present field value to less than one-quarter of this value (Fig. 3). The modified Thellier technique is specifically designed to detect alteration during laboratory heating, and samples that were significantly altered before collection would not be expected to yield high-quality palaeointensity results, such as are observed here (Fig. 3c). In addition, recent transmission electron microscopy results indicate that submicrometre-sized magnetite encased in fresh glass remains unoxidized even in samples as old as 32 Myr²⁰. We therefore regard these palaeointensity determinations as an accurate measure of the ambient field intensity when these surface flows initially cooled. Because the crystalline interiors of the basalt flows acquire a thermoremanence in this same field, we expect that their magnetizations will also reflect these field fluctuations, in addition to variations related to sample geochemistry¹⁵ and possibly alteration²¹.

The pattern of intensity variation from our glass samples (Fig. 2b) is remarkably similar to that documented from the independent palaeointensity records over the past 50 kyr. Field intensities comparable to the present field value are found at the ridge axis, whereas peak intensity values are 1–2 km off-axis and much lower values (~10% to 25% of the present field) occur further from the axis. A similar trend is evident in earlier palaeointensity results using a smaller number of samples on the northern EPR¹⁸. The pattern of palaeointensity on individual profiles also reveals many of the features visible in the near-bottom magnetic anomaly data (see Supplementary Information). For example, the profile from 19.75° S shows low values (near the present field intensity) at the axis, flanked by higher values 1–2 km from the ridge and significantly lower values further off-axis. Similarly, the asymmetric anomaly pattern from the 19.41° S profile is paralleled by the palaeointensity values from the surface flows. Closer inspection, however, reveals that the extent of the high palaeointensity values is significantly broader than the peak in the corresponding magnetic anomaly profile. This apparent discrepancy is readily explained by the observation that recent flows on the EPR may occur a few kilometres from the locus of spreading, either through off-axis eruptions²² or downslope redistribution of lava from the axis²³. Given this lava emplacement distribution, both the magnitude and pattern of the observed palaeointensity data provide evidence that large geomagnetic intensity fluctuations are recorded in the oceanic crust.

Comparison of glass sample palaeointensities with independent estimates of past dipole intensity fluctuations (Fig. 2) provides a means for assessing the age of these flows, and hence quantifying the lateral extent of off-axis flows. Although past geomagnetic intensity variations are incompletely known, the pattern since 50 kyr ago is sufficiently distinctive to be used as a dating tool. Intensities significantly above the present field value (for example, ~1 km east of axis on the 19.75° S profile) most probably represent lavas that erupted in the interval from 1–3 kyr ago. An equally powerful constraint is provided by the distribution of flows with low intensities near 2×10^{22} Am². These extremely low values most

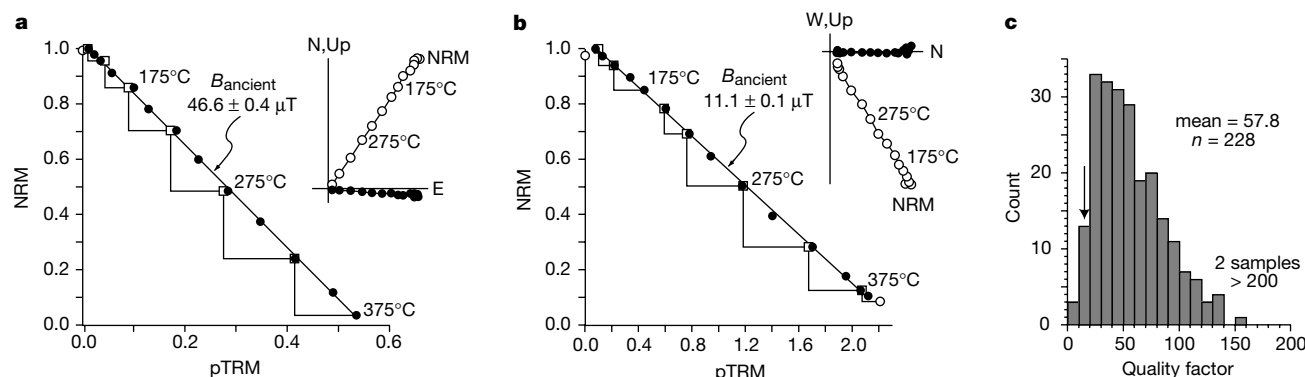


Figure 3 Representative absolute palaeointensity results. They show: **a, b**, a range of field values; and **c**, overall data quality (quality factor, q ; ref. 19) of EPR samples. Graphs portray stepwise decay of natural remanent magnetization (NRM) versus acquisition of partial thermoremanence (pTRM), both normalized by the initial NRM value. Insets show decay of magnetization vector from zero-field heating steps. Filled (open) circles are projections of the magnetic vector onto horizontal (vertical) planes. The laboratory field was $25 \mu\text{T}$. Data must pass the following reliability tests: (1) The best-fit line through the NRM/pTRM points must have low scatter, with $\sigma/|b|$ (the standard error for the slope normalized by the slope¹⁹) less than 0.1. (2) Over the temperature interval used for the slope calculation, the maximum deviation of any pTRM check from the best-fit line must be less than 10% of the length of this NRM–pTRM segment. (3) The component of magnetization used for the palaeointensity determination must trend to the origin. The angular difference (α) between a principal component³⁸ determined from the NRM points

tied to the origin and free from the origin must be less than 10° . (4) The maximum angular deviation (MAD) of this component must also be less than 10° . In addition, we have discovered that the reagent grade NaCl used to encase the glass chips during the Thellier experiment occasionally contains ferromagnetic material that may gain a sufficient TRM to compromise some palaeointensity results. In order to screen for this contamination, we require that the final in-field remanence step for the pellet agree within 25% of the remanence of the glass chip alone after dissolution of the pellet. Arrow in **c** indicates average quality factor for subaerial lava flows from Hawaii¹⁹. **a**, pagc40d; $f = 0.940$; $g = 0.899$; quality factor $q = 90.3$; $\sigma/|b| = 0.009$; $\alpha = 0.4$. **b**, pagc29b; $f = 0.861$; $g = 0.906$; $q = 93.7$; $\sigma/|b| = 0.008$; $\alpha = 3.0$. B_{ancient} is the ancient field intensity, f is the NRM fraction and g is the gap factor as defined in ref. 19. For locations of samples pagc40d and pagc29b see Supplementary Information.

plausibly are associated with flows erupted during or near the Laschamp excursion (Fig. 2a), where comparable or lower intensities have been documented^{24,25}. Based on an age of 46.6 ± 2.4 kyr for the Laschamp excursion²⁵ and assumed constant spreading, these low intensities should occur ~ 3 km from the axis. The very low intensities ~ 4 km west and 4.9–5.8 km east of the ridge on profile 19.71° S suggest that these flows were displaced or possibly erupted ~ 1 km and 1.4–2.3 km from the axis, respectively. Similar arguments imply a somewhat larger displacement (1.6–3.0 km) on the west side of the profile at 17.38° S.

One puzzling aspect of the palaeointensity results is the apparent lack of high intensities off-axis where such values might be expected from a peak (at ~ 55 kyr ago) in the sedimentary record. Our sampling recovered a single high value on the eastern flank at EPR at 17.38° S, but near-bottom side-scan sonar images suggest that this flow may be a recent off-axis eruption. Although the high degree of correspondence between the sedimentary palaeointensity and crustal magnetization records over the entire Brunhes (Fig. 1) supports a geomagnetic origin for the features common to both records, neither constitutes a perfect record of geomagnetic field behaviour. Several aspects of the sedimentary composite palaeointensity record⁴, including the arbitrary normalization of records to unit mean over the interval 24–56 kyr ago and the relatively poor time control resulting from the lack of clarity of marine isotopic stage 3, may contribute to uncertainty in the amplitude of the stacked sedimentary record near 55 kyr ago. No significant peak near 55 kyr ago is evident in either the magnetic anomaly or magnetization inversion solution stacks (Fig. 1). Until additional absolute palaeointensities from this time interval are available, we suggest that the crustal magnetization variations are as likely as the sedimentary data to reflect the true geomagnetic intensity within this interval.

Crustal accretion and the origin of the axial magnetic high

The near-bottom magnetic anomaly data, together with palaeointensity estimates and geochemical data from surficial glass samples, provide important constraints on crustal accretionary processes at the ultrafast spreading southern EPR. Seismic observations from the EPR reveal that layer 2A, commonly interpreted as the lithological boundary between extrusives and sheeted dykes^{26,27}, thickens by about a factor of two within a few kilometres of the ridge either through eruptions off-axis or redistribution and ponding of axial flows^{22,23}. If seismic layer 2A is equivalent to the extrusive magnetic source layer, its thickening can be reconciled with the presence of the CAMH only if the magnetization of the extrusive layer decreases by a significant factor (3–5) over distances comparable to the thickening of seismic layer 2A²¹.

Although rapid alteration was initially suggested as the dominant mechanism for this magnetization reduction²¹, more recent studies^{28,29} have called into question the importance of alteration over timescales relevant for the CAMH. Our new results suggest that geomagnetic intensity variations are sufficient to account for the magnetization contrast generating the CAMH. Inversions of near-bottom anomalies at 19.75° S, both with a constant-thickness source and with a thinner axial source, yield magnetization patterns that closely resemble fluctuations in glass palaeointensities (Fig. 4). Since magnetic anomalies are the linear sum of contributions from the entire magnetic source layer, the discrepancy between the narrow magnetization peak and the glass palaeointensities also implies that the lavas displaced from the axis constitute a volumetrically minor portion of the extrusive layer, but these standard inversions assume that magnetization does not vary with depth and so are not ideal if the pattern of crustal accretion is more complex.

To understand the origin of the CAMH better and to assess the volumetric significance of extrusives emplaced away from the axis, we generated stochastic models of lava emplacement²³ simulating various patterns of off-axis addition to the extrusive layer. The

magnetization in these models is proportional to the palaeofield intensity at the time of lava emplacement (Fig. 2a) and the average FeO* content¹⁵ of the lavas (compare ref. 30). We examine three models: (1) a constant-thickness (0.5 km) source layer with no off-axis emplacement; (2) a variable-thickness source layer with a distribution of off-axis flows matching the pattern of seismic layer 2A; and (3) a variable-thickness source layer with a smaller volume of off-axis flows whose extent is consistent with our surface glass palaeointensity results.

Results of these emplacement models for profiles at 19.75° S and 17.38° S (Fig. 5) indicate that the volumetric importance of off-axis flows is variable, but may be significantly less than inferred from seismic data on the EPR. Although no seismic data are coincident with the 19.75° S profile, nearby results suggest that layer 2A may double in thickness within 0.7–1.5 km of the axis³¹. Models with this average pattern of thickening have a low-amplitude CAMH and an overall pattern similar to that expected from topographic variations and a constant magnetization. A model with 20% off-axis flows (Fig. 5a) provides a closer match to the observed anomaly data. A simple model with a constant-thickness source layer (and no off-axis flows) arguably provides the best fit to the observed data, matching the overall amplitude of the CAMH as well as the older anomaly fluctuations. Seismic data coincident with the profile at 17.38° S document a threefold increase in layer 2A thickness within ~ 3 km of the axis³². A model that matches this pronounced off-axis

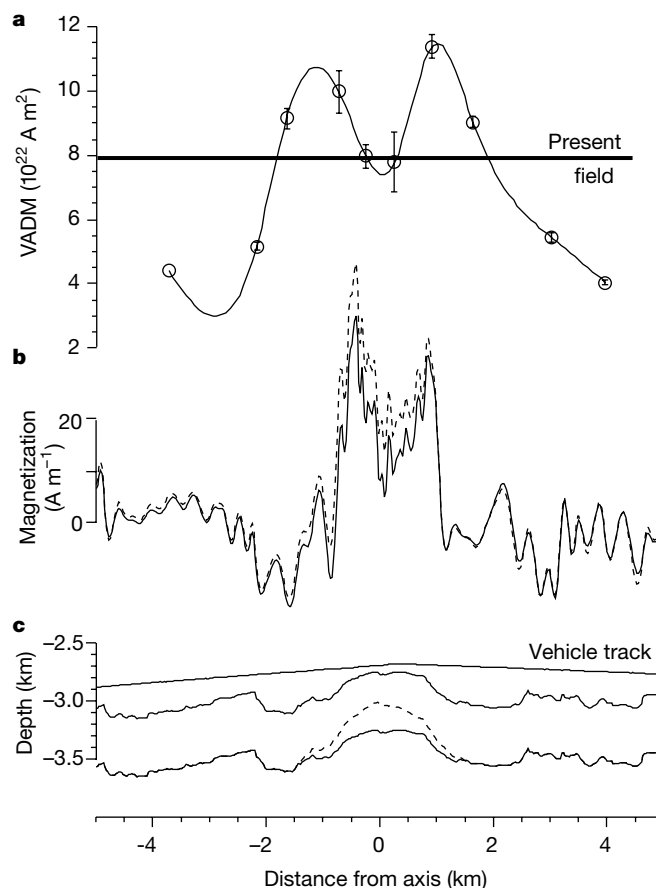


Figure 4 Similarity of glass palaeointensities and inversion solution magnetization for profile at 19.75° S. Comparison of glass palaeointensities (a) and magnetizations derived from inversion of near-bottom magnetic anomalies (b). c, Source-layer geometries used in the inversions. Constant-thickness (solid line) and variable-thickness (dashed line) sources approximating off-axis doubling of layer 2A thickness inversions indicate that a lower magnetization is required on axis, with flanking areas of high magnetization. Curve connecting palaeointensity data is a spline fit.

thickening (70%) yields a poor match to the observed anomaly, as do the high-amplitude anomalies generated by a constant-thickness model (Fig. 5b). However, a lesser amount of off-axis extrusive thickening is necessary to model the low amplitude CAMH on this profile, and a model with about 25% of the extrusive layer contributed by off-axis flows yields a reasonable match.

Applications of palaeointensity data

Our results illustrate the potential of combining near-bottom magnetic anomaly data and sampling to address important aspects of ridge crest accretion as well as geomagnetic field behaviour. The large dipole intensity variations over the past 50 kyr reflected in absolute palaeointensities provide a new dating tool for young mid-ocean ridge basalts on timescales difficult to assess with radiometric techniques. In addition to general age constraints provided by high field values at 1–3 kyr ago and extremely low intensities at ~40 kyr ago, even tighter age limits should be possible by comparison of absolute palaeointensities of the youngest axial flows with historical measurements of the geomagnetic field³³. For example, the large (~25%), approximately linear decrease in intensity over the past

200 years is ideally suited for dating axial eruptions that are presumed to recur on timescales of 5–100 years^{34,35} on fast spreading ridges such as the portion of the EPR studied here. Regardless of the absolute age, the spot readings of the geomagnetic field recorded by quenched glass provide a powerful age discriminant for seafloor basalts, particularly when combined with geochemical data. Our results illustrate the role of off-axis thickening of the extrusive layer in controlling the amplitude of the CAMH. More detailed information on the pattern of lava accumulation may ultimately be possible as the pattern of past geomagnetic intensity variations is refined and if geochemical variations can be adequately documented. Finally, the overall similarity of near-bottom magnetic anomaly data and sedimentary relative palaeointensity records throughout the Brunhes demonstrates that geomagnetic intensity is primarily responsible for the coherent magnetization variations within the Brunhes. The recognition of a geomagnetic signal on this extremely fast-spreading ridge provides additional temporal markers for correlation and dating within the Brunhes, and suggests that seafloor records may constitute a valuable source of information on past geomagnetic field fluctuations. □

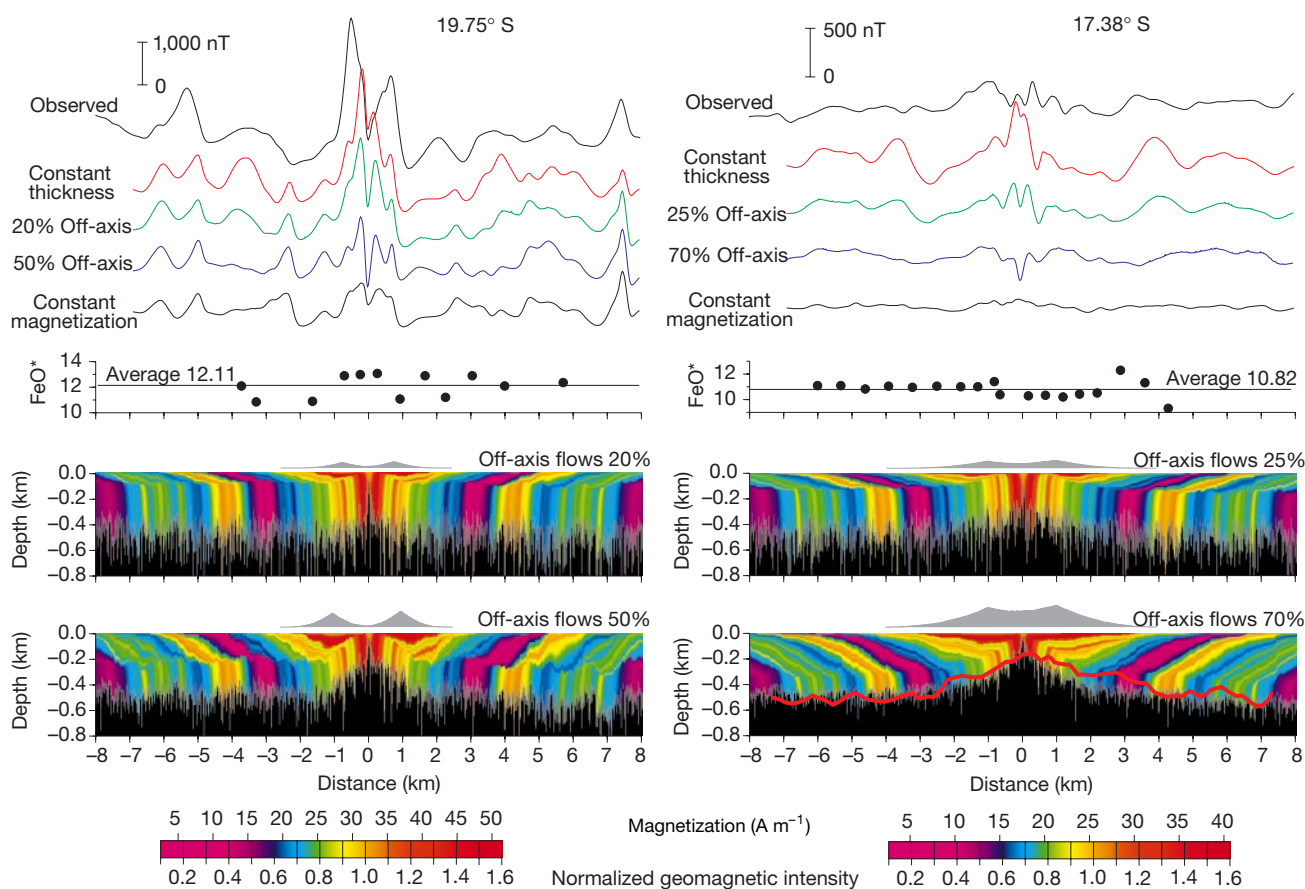


Figure 5 Lava emplacement models and associated magnetic anomaly profiles for profiles at 19.75° S and 17.38° S on the East Pacific Rise. A bimodal emplacement model based on that of ref. 23 was used to generate lava distributions simulating off-axis thickening of extrusive layer as inferred from seismic observations. Lower models show distributions that qualitatively match seismic thickening patterns inferred for 19.75° S (see text) and observed at 17.38° S (thin red line; ref. 32). Upper models show lava emplacement models with smaller volumetric contributions from off-axis flows that yield better agreement with the observed anomalies. Stochastic models all incorporate short on-axis flows with a half width of 100 m and dyke emplacement with a standard deviation of 10 m (ref. 23). The distribution and relative volume of off-axis flows is shown above each model. The magnetization of lavas is proportional to both palaeointensity (heavy line

in Fig. 2a) and the average FeO* content of the surficial samples (filled circles). The relationship between FeO* and remanence is that derived from zero-age samples on the southern EPR¹⁵. Colour variations correspond to isochrons in the extrusives; dykes are shown in black. Magnetic anomalies from these models were calculated from multiple strips through the model. All models used the observed bathymetry and observation path (see Supplementary Information). Magnetic anomalies corresponding to the lower (upper) stochastic models are shown as blue (green) lines. The anomaly resulting from a constant thickness (0.5 km) source with magnetization proportional to palaeointensity (heavy line in Fig. 2a) is shown as red line. Magnetic anomalies resulting from a constant magnetization (20 A m⁻¹) source (lower black line) are shown to illustrate effect of topography.

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Symmetry-induced formation of antivortices in mesoscopic superconductors

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Recent progress in nanotechnology has stimulated interest in mesoscopic superconductors as components for quantum computing and cryoelectronics. The critical parameters for superconductivity (current and field) of a mesoscopic sample are determined by the pattern of vortices in it, which in turn is controlled by the symmetry imposed by the shape of the sample (see ref. 1 and references therein). Hitherto it has been unclear what happens when the number of vortices is not consistent with the natural symmetry. Here we show that additional vortex–antivortex pairs nucleate spontaneously so as to preserve the symmetry of the sample. For example, in a square with three vortices, the spontaneously generated pair, along with the original three vortices, distribute themselves so that the four vortices sit in the four corners, with the antivortex in the centre. The measured superconducting phase boundary (of superconducting transition temperature T_c versus magnetic field strength) is in very good agreement with the calculations, giving direct experimental evidence for these symmetry-induced vortex–antivortex pairs. Vortex entry into the sample is also changed: vortices enter a square in fours, with antivortices generated to preserve the imposed vorticity. The symmetry-induced nucleation of antivortices is not restricted to superconductors, but should also apply to symmetrically confined superfluids and Bose–Einstein condensates.

The nucleation of superconductivity is normally analysed in terms of the linearized Ginzburg–Landau (LGL) equation²

$$\frac{1}{2m^*} \left(-i\hbar\nabla - \frac{2e}{c}\mathbf{A} \right)^2 \psi = -\alpha\psi \quad (1)$$

with $\mathbf{H} = \text{rot}\mathbf{A}$ the applied magnetic field. Equation (1) looks like an ordinary Schrödinger equation for a free particle in an external magnetic field, where the ‘wavefunction’ ψ stands for the complex superconducting order parameter and $\alpha = -\hbar^2/2m^*\xi^2(T)$, with $\xi(T)$ the temperature-dependent coherence length. The superconducting boundary condition³ considerably complicates the solution of equation (1); we used a recently developed analytical gauge transformation, such that the vector potential $\mathbf{A}$ has no component normal to the sample boundaries of arbitrary regular polygons (L. F. C. *et al.*, unpublished work). The lowest Landau level of equation (1) then gives the shift of the superconducting transition temperature $T_c(H)$.

The solutions of the LGL equation for the square, shown in Fig. 1, are characterized by irreducible representations (irreps) A , B , E_- and E_+ , with the characters $\exp(in\pi/2)$, for $n = 0, 2, -1$ or 1 under the fourfold rotation, respectively⁴. The ground Landau level shows an oscillatory cusplike behaviour as a function of flux, corresponding to a crossover of states belonging to different irreps (Fig. 1a).

Figure 1b shows the comparison of the calculated and the measured phase boundary (open squares) for the mesoscopic Al square. The theoretical coloured curve is obtained from Fig. 1a, where the ground state level is selected for all flux values. The $T_c(H)$ boundary is measured resistively, using an electronic feedback circuit. For experimental details, we refer to ref. 5. The agreement

between the calculated lowest Landau level and the measured $T_c(\Phi)$ is very good. We note that no fitting parameters were needed to match the cusp positions.

At the cusp positions on the phase boundary the vorticity L changes by one, starting from zero (no fluxoids) at low magnetic fields. In the case of a disk (C_∞) the vorticity is just the orbital quantum number, L , defining the flux, $L\Phi_0$, carried by the giant

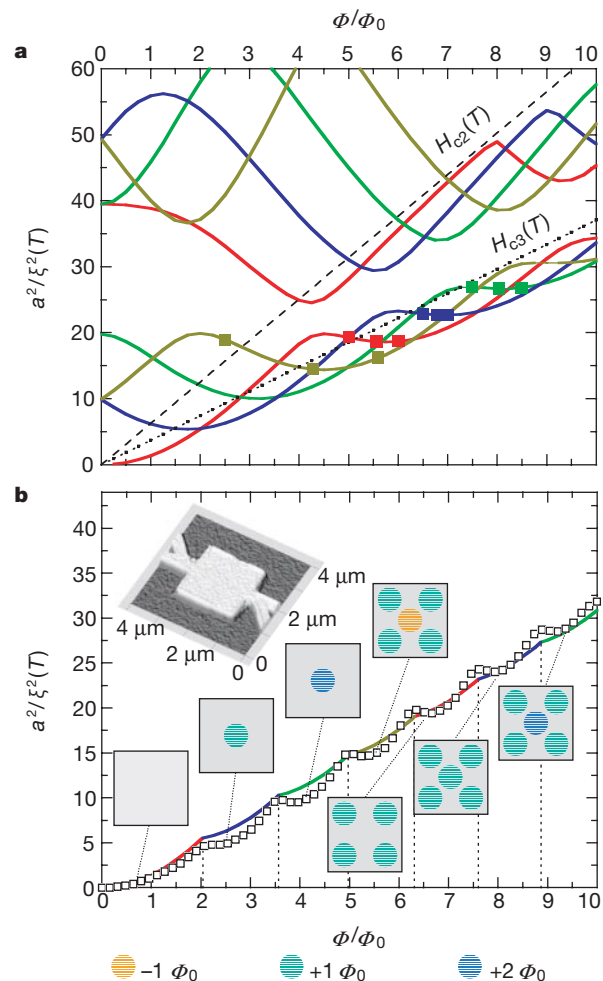


Figure 1 Calculated and measured superconducting $T_c - H$ phase boundary for a square. **a**, Lower eigenvalues of the LGL equation for the mesoscopic square, as a function of magnetic flux Φ/Φ_0 , with superconductor–vacuum boundary conditions. The different colours correspond to the four irreducible representations (irreps) A (red), B (green), E_+ (dark blue) and E_- (dark yellow). Because of the discrete C_4 symmetry there is a ‘repulsion’ of the levels, giving a regular pattern of avoided crossings between levels belonging to the same irrep. The flux is defined as $\Phi = Ha^2$, with a the side length of the square, and H the applied magnetic field. The superconducting flux quantum $\Phi_0 = h/2ec$. On the vertical axis, the critical temperature T_c is linearly decreasing with increasing $a^2/\xi^2(T)$. The coloured squares on the lower lines indicate the values of Φ for which vortex patterns are shown in Figs 2–5. The dashed straight line is the upper critical field H_{c2} in a bulk type 2 superconductor, and $H_{c3} = 1.69 H_{c2}$ (dotted straight line) is the surface critical field for a semi-infinite slab bordered by a straight superconductor–vacuum interface. **b**, Comparison between the calculated (continuous coloured curve) and the measured $T_c(\Phi)$ phase boundary (open squares). The experimental data have been corrected for the presence of the measuring leads. A zero-temperature coherence length $\xi(0) = \xi(T)\sqrt{1 - T/T_c} = 99$ nm was used to obtain the best agreement. The zero-field critical temperature is $T_c = 1.36$ K. The top left inset shows the atomic force microscope image of the investigated $2 \times 2 \mu\text{m}^2$ square. In the seven insets, the vortex structure in different regions of the phase diagram is shown schematically with coloured circles. In the range $5 < \Phi/\Phi_0 < 6.3$, an antivortex is formed spontaneously at the centre of the square, coexisting with four Φ_0 -vortices along the diagonals.

vortex⁶. For the square the rotational axis is of finite order (C_4) and, therefore, the distribution of vortices in symmetry-consistent solutions, considered here, is not *a priori* evident. The seven insets in Fig. 1b show schematically the distribution of vortices, which are clearly different from the giant vortex states.

In the case of small L values, vortices can occupy one central and four diagonal positions. In contrast to the diagonal vortices which always enclose a single quantum Φ_0 , the central vortex can have different winding numbers in order to conserve the total vorticity of a given state. The contribution of the two kinds of vortices (central + four diagonal) to the total winding number of the states shown in Fig. 1b is given by

$$L = n + 4m \quad (2)$$

where $n = 0, 1, 2$ or -1 and $m = 0$ or 1 .

First, the nature of the central vortex changes, whenever vorticity is changed by one. Thus, the central vortex is absent in the first state, is a Φ_0 -vortex in the second state, is a giant vortex in the third state and is an antivortex (the winding number is negative) in the fourth state (see Fig. 1b). Second, the sequence of winding numbers of the central vortex ($-1, 0, 1, 2$) is periodically repeated when going to the right of the phase diagram. Because the kinetic energy of a vortex is proportional to L^2 , the system prefers to split the giant vortex into a sum of smaller vortices⁷ if there are no special symmetry restrictions. This explains why only four numbers mentioned above appear as winding numbers for the central vortex. On the other hand, the formation of antivortices is dictated completely by the discrete symmetry. Indeed, in the state with $L = 3$, one cannot distribute three Φ_0 -vortices on the square keeping the symmetry. The dilemma is solved by having four Φ_0 -vortices and adding one antivortex in the centre. This is also the lowest energy state

(compare with the fourth state in Fig. 1b).

Spontaneous generation of antivortices also controls the flux penetration into mesoscopic superconductors. Our theoretical analysis shows that in regular polygons with N edges the flux enters always by $N\Phi_0$ -vortices through the edge centres, because these are the symmetry points with the lowest values of $|\psi|$ on the borders. Increasing the field further, it is energetically favourable for these vortices to reorient towards the corners of the polygon (Fig. 1b), thus paving the way for the entrance of the next set of $N\Phi_0$ -vortices. However, such a reorientation cannot be performed by a continuous rotation of the vortex patterns, as that would violate the imposed symmetry. Therefore, the formation of additional antivortices and vortices turns out to be indispensable.

This is further illustrated for the square. Three states in the evolution of the vortex patterns—entrance of vortices (initial), the transient state (intermediate), and the formation of diagonal vortices (final)—are shown in Figs 2–5 for the four irreps. The dynamics of this transformation differs dramatically for states of different symmetry. Thus, in the case of irreps E_+ and A four vortices and four antivortices arise in the intermediate state. For the other two irreps the intermediate state is associated with the change of the winding number of the central vortex: the giant $2\Phi_0$ -vortex decays into a giant antivortex $-2\Phi_0$ and four diagonal Φ_0 -vortices in the case of irrep B , whereas for the irrep E_- the central Φ_0 -antivortex is transformed into a giant $3\Phi_0$ -vortex by absorbing the four lateral Φ_0 -vortices. For direct visualization of these unusual vortex patterns (Figs 1–5), local vortex-imaging techniques, such as scanning Hall probe^{8,9}, scanning tunnelling^{10,11}, magnetic force¹², and Lorentz microscopy¹³ are very promising.

The appearance of symmetry-induced antivortices remains valid for all other superconducting polygons (triangle, pentagon, and so on). Our results might also be applicable to large antidot arrays, where the spontaneous generation of antivortices could provide conditions for stronger vortex pinning. The spontaneous generation of antivortices is clearly a fundamental property of symmetrically confined vortex matter in general. Our findings are applicable not only to superconductors, but also to superfluids (^4He and ^3He) and Bose–Einstein condensates. Superfluids rotated in a triangular or

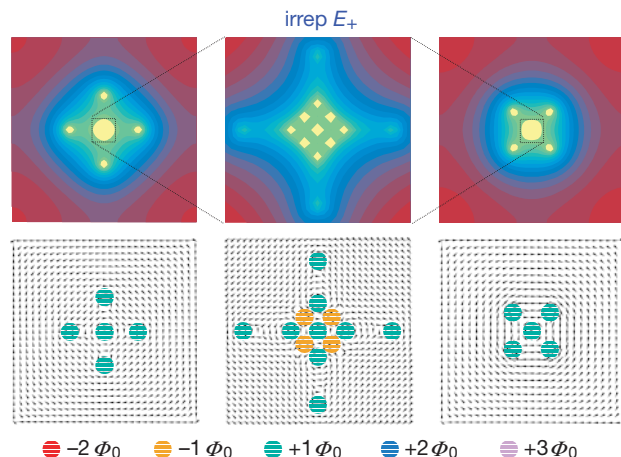


Figure 2 Vortex entry into a square for applied magnetic fields generating five flux quanta. The symmetry of the corresponding states is E_+ (blue curves in Fig. 1). The top panels show the density of the order parameter $|\psi|^2$ for the initial, intermediate and final stages corresponding to the Φ values marked by the squares (from left to right) in Fig. 1a. The highest density is shown in red. The lowest $|\psi|^2$ values are coloured yellow, indicating the positions of vortices and antivortices. The lower three panels show the phase gradient of the order parameter, corresponding to the above contour plots. The gradient is represented by arrows with appropriately scaled lengths. The coloured filled circles in the lower three panels relate each vortex to its winding number (see below). Here, in the middle panels the central region of the square was magnified 16 times. Stage one (on the left) shows the four 'lateral' vortices approaching the central vortex from the middles of the square's sides. At the transition state (in the centre), the central region suddenly changes into a chequerboard-like pattern of vortices and antivortices. In a subsequent step (not shown here) the vortices from the four pairs of lateral vortices approach each other and suddenly 'rotate' by 90° . As the flux further increases, the antivortices and the 'rotated' Φ_0 -vortices move towards the corners and eventually merge to form single diagonal Φ_0 -vortices (on the right).

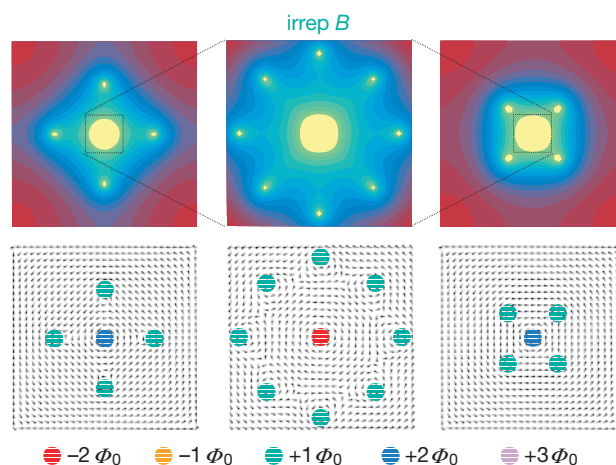


Figure 3 Vortex entry into a square for applied magnetic fields generating six flux quanta. The symmetry of the corresponding states is B (green curves in Fig. 1). The same colour conventions as in Fig. 2 were used. The middle plots correspond to the central region of the square magnified 5 times. The reorientation mechanism in this case involves the transformation of the central $2\Phi_0$ giant vortex (on the left) into a giant antivortex ($-2\Phi_0$ -vortex) and four diagonal Φ_0 -vortices (central panels). Under this transformation the total vorticity remains invariant: $8 \times \Phi_0 - 1 \times 2\Phi_0 = 6\Phi_0$. While the diagonal vortices move to the corners, the lateral Φ_0 -vortices further approach the centre and are absorbed by the central antivortex, restoring in this way the initial $2\Phi_0$ giant vortex, characteristic for the irrep B .

square vessel would also generate antivortices in order to comply with the imposed symmetry. By proper arrangement of the laser fields, the vortex patterns in Bose–Einstein condensates confined by triangular or square traps could also reveal symmetry-induced antivortices.

Our symmetry-conserving results for a triangle form a natural generalization to superconducting boundary conditions of the quantum-mechanical problem of a “particle in an equilateral triangle”¹⁴. An intriguing correspondence can be drawn between

the eigenstates in the triangle and families of leptons (electrons or muons) and quarks. □

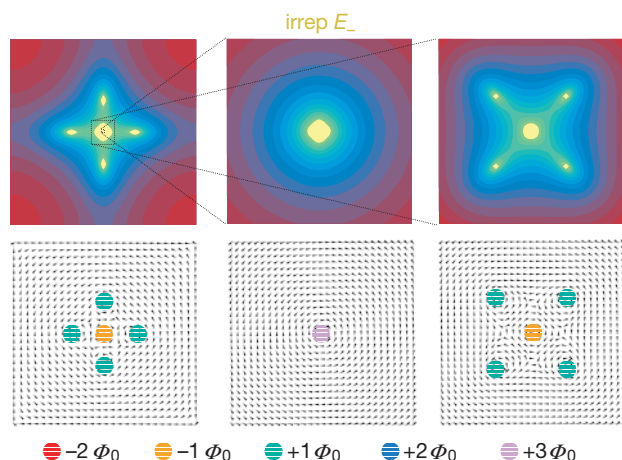


Figure 4 Vortex entry into a square for applied magnetic fields generating three flux quanta. The symmetry of the corresponding states is E_- (dark yellow curves in Fig. 1). The same colour conventions as in Fig. 2 were used. The central region of the square is magnified 64 times, for the middle plots, and 8 times for the plots on the right. In this mode, the lateral Φ_0 -vortices approach and merge with the central antivortex to form a giant $3\Phi_0$ -vortex. With increasing field, the $3\Phi_0$ -vortex will split into four diagonal Φ_0 -vortices and the initial single antivortex (on the right). In order to be able to see the four separate Φ_0 -vortices, the panels on the right had to be magnified. This is owing to the strong attraction of the Φ_0 -vortices to the central antivortex. For the same reason, the spacing between the three dark yellow squares in Fig. 1a is large, as compared to the other irreps.

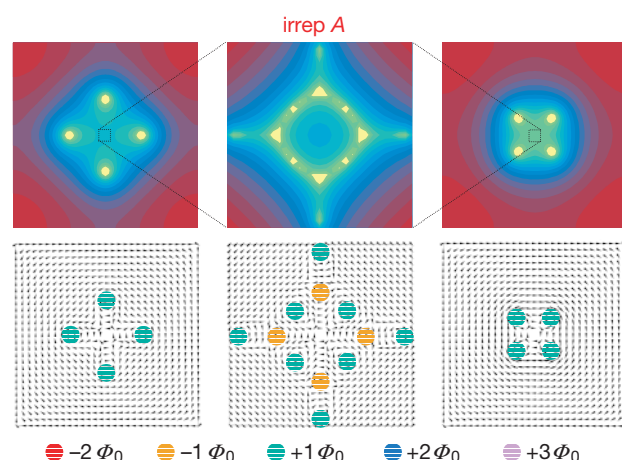


Figure 5 Vortex entry into a square for applied magnetic fields generating four flux quanta. The symmetry of the corresponding states is A (red curves in Fig. 1). The same colour conventions as in Fig. 2 were used. The middle plots correspond to the central region of the square magnified 16 times. In the initial stage (left), there is no central vortex in this case. The approach of the lateral Φ_0 -vortices induces the creation of a central vortex–antivortex pattern (central panels), which is however rotated by 45° , as compared to Fig. 2. At higher flux (on the right), the lateral vortices and antivortices will annihilate, leaving four diagonal Φ_0 -vortices which move towards the corners.

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Flexible filaments in a flowing soap film as a model for one-dimensional flags in a two-dimensional wind

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The dynamics of swimming fish and flapping flags involves a complicated interaction of their deformable shapes with the surrounding fluid flow. Even in the passive case of a flag, the flag exerts forces on the fluid through its own inertia and elastic responses, and is likewise acted on by hydrodynamic pressure and drag. But such couplings are not well understood. Here we study these interactions experimentally, using an analogous system of flexible filaments in flowing soap films. We find that, for a single filament (or ‘flag’) held at its upstream end and otherwise unconstrained, there are two distinct, stable dynamical states. The first is a stretched-straight state: the filament is immobile and aligned in the flow direction. The existence of this state seems to refute the common belief that a flag is always unstable and will flap^{1,2}. The second is a flapping state: the filament executes a sinuous motion in a manner akin to the flapping of a flag in the wind. We study further the hydrodynamically coupled interaction between two such filaments, and demonstrate the existence of four different dynamical states.

Experiments that cleanly reveal the nature of interactions between a deformable body and a flow are difficult to perform, with complications including the control of three-dimensional flow effects and the challenge of flow visualization. Soap film is a convenient experimental system that resembles two-dimensional hydrodynamics in many respects^{3–7}. In this experiment, we take advantage of the relative simplicity of this system, and use the flowing soap film as a laminar two-dimensional flow tunnel. The experimental set-up is shown in Fig. 1. The horizontal span of the tunnel is 8.5 cm, the typical film thickness 3–4 μm , and flow speeds 200–300 cm s^{-1} (see Methods). The experiment is performed 80 cm below the flow entrance, at the midline. The filaments are 0.15 mm in diameter and 2–6 cm long. Their linear density is about $2.0 \times 10^{-4} \text{ g cm}^{-1}$. The rigidity of the filament is estimated to be the order of 0.1 erg cm, tested in a steady soap film where the deflection is measured under a known force. The fluid wets the filament, with surface tension forces constraining the filament to lie always in the plane of the film. The length of the filament can be changed incrementally through the holder at the upstream end, which abuts the film perpendicularly.

At a fixed flow rate, there exists a critical filament length L_c , below which the filament is stretched straight and aligned with the flow. Regardless of the magnitude of an externally imposed disturbance, after a few oscillations, the filament quickly returns to this rest state. If, however, the length of the filament exceeds L_c , the system becomes bi-stable, and two different stable dynamical states are observed. One remains the stretched-straight state, as when $L < L_c$. Figure 2a shows such a state (exposure time 0.5 ms). Using a monochromatic light source, the photograph shows instantaneous interference patterns that reveal small thickness variations. A narrow von Kármán vortex ‘street’^{8,9} (that is, a train of vortices of alternating signs) can be seen trailing behind the filament. We note

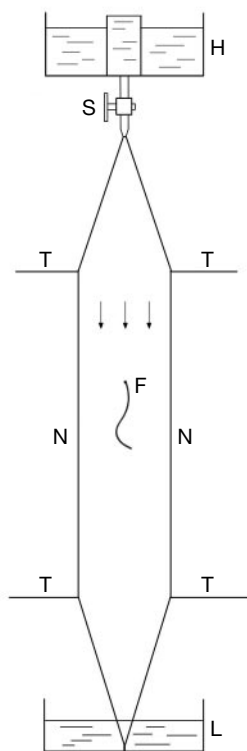


Figure 1 Experimental set-up. A vertical soap film is fed from the top (H) with a solution of 1.5% Dawn, a commercial brand of dishwashing liquid soap, in distilled water. The pressure head is fixed using an overflow mechanism. The attached nozzle (S) regulates the flux. Two nylon threads under tension (T), spread from the nozzle, run parallel, and then converge into the receiving container (L). The upper end of the silk filament (F) is fixed using a thin tube, which is introduced normal to the film plane.

that this street is shed starting from the free end rather than from the top, where the flow first encounters the filament. Despite the oscillations of the street, the filament remains a static, seemingly rigid body. Indeed, transient small disturbances (such as a small displacement at the free end, or a thin vortex wake produced by a cylinder placed upstream) do not destroy the stability of this state.

However, if the external perturbation is sufficiently large, the filament jumps to a stable flapping state (Fig. 2b), whose persistence is quite robust to perturbation. Unlike a simple pendulum, the undulation is well fitted by a travelling harmonic wave with a spatially varying envelope:

$$y(x, t) = f(x)\sin(2\pi\nu t + 2\pi x/\lambda)$$

Here, $y(x, t)$ is the horizontal displacement of the filament from the centre-line, measured at a vertical distance x from the fixed point for time t . $f(x)$ is a spatial envelope function (increasing monotonically from the fixed point), ν is the flapping frequency and λ the wavelength. When the filament length is about 3 cm long and flow speed 280 cm s^{-1} (the corresponding Reynolds number is of the order of 10^4), we observed a typical flapping frequency of about 50 Hz and an amplitude (the total excursion of the free end) of about 1.5 cm. A thin vortex street is still evident, but is strongly modified by the large-scale flapping motion. The successive small eddies produced by a single stroke are now of a single sign, unlike the alternating signs in the stretched-straight case. This local vortex

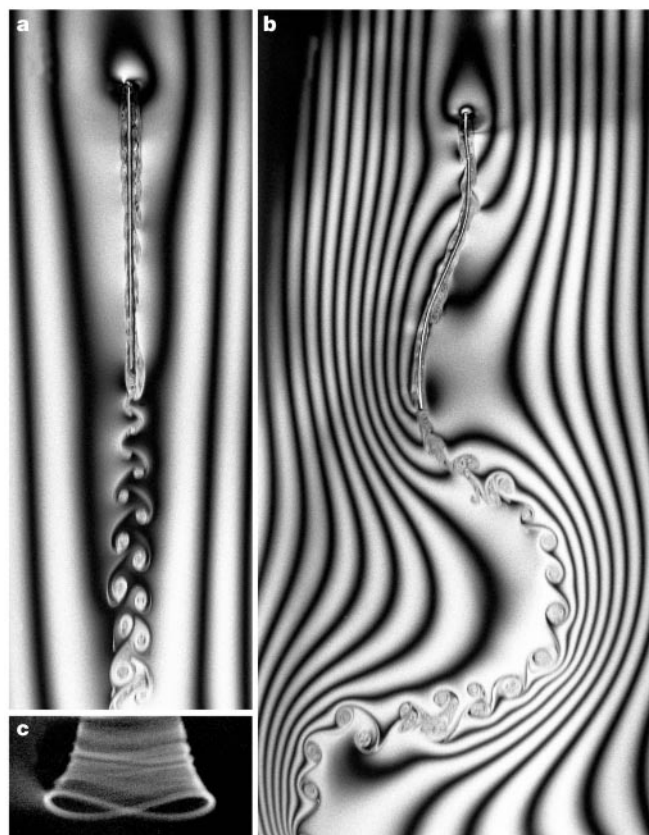


Figure 2 The two stable states of the filament. When the filament length is greater than the critical value, it can stay in either a stretched-straight state or an oscillatory state. The flow is visualized using interference patterns under a sodium lamp. **a**, The filament is stretched straight in the flow. Von Kármán type vortices are shed from its free end. **b**, The oscillatory flapping state. Flow structures, modulated by the filament, are advected downstream. Interference fringes run in the flow direction, indicating that the soap film is under little stretching and compression. **c**, The free end of the filament shows a ‘figure of eight’ trajectory owing to the existence of the travelling waves associated with the flapping motion.

structure resembles that resulting from a Kelvin–Helmholtz instability¹⁰, suggesting that each stroke and counter-stroke produces a shear-layer of alternating sign. The resulting vortical field does not develop eddies of a size comparable to the flapping amplitude. This is significantly different from a von Kármán street behind a cylinder, or the wake generated by the tail of a

flapping fish (ref 11 and ref. 12 and references therein), where one observes vortices of a size comparable to the body. In Fig. 2c, the lower part of the filament is shown in a 0.5-s exposure. Because of the travelling wave and the inextensibility of the filament, the free end executes a ‘figure of eight’ trajectory.

Although the flapping state is stable, the filament can be forced to return to the stretched-straight state by briefly holding straight the filament (such as with a pair of tweezers), or by shortening it until L is brought below L_c . Both stretched-straight and flapping states can be alternately selected, either by externally forcing the filament or by exploiting the dynamical hysteresis.

If L is sufficiently large, the stable stretched-straight state disappears, and only the flapping state remains. It is difficult to determine experimentally whether this is due to a loss of linear stability at some finite L , or is due to finite noise in the flow being sufficient to overcome a barrier between the two stable states. Overall, the observed phenomenon suggests the existence of a sub-critical bifurcation (see ref. 13 for example).

For a very long filament ($L > 6$ cm), the observed flapping motion seems to be limited to within about two wavelengths of the free end. This suggests that the tension exerted on the filament, arising from viscous shear stress exerted by the flowing fluid, as well as from the weight of the filament, constrains the motion; the flapping motion thus becomes an edge effect.

Figure 3 shows the experimental measurements of the flapping amplitude and frequency as functions of the filament length. In the measurements, the free stream flow speed is fixed, and the length of the filament is changed incrementally. The amplitude of the flapping motion, measured as the maximum flapping span, changes with length (Fig. 3a). The frequency is essentially constant as a function of length (Fig. 3b).

An important generalization to consider is the dynamics of two ‘compliant walls’ that interact with each other through the flowing fluid. This is an open-flow analogue to the dynamics of a compliant pipe¹⁴. When two identical filaments are inserted side by side into the flowing soap film, cooperative dynamical states are observed. As shown in Fig. 4 (fixing $L = 3.6$ cm and $U = 220$ cm s⁻¹), if the inter-filament distance d is sufficiently small ($d/L < 0.21 \pm 0.04$), the filaments tend to phase lock and flap in phase with each other

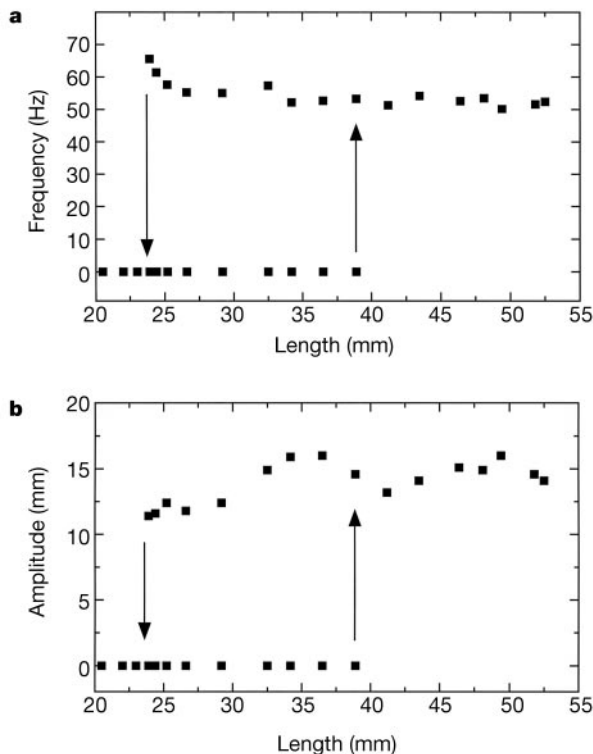


Figure 3 The flapping frequency and amplitude of the filament as functions of L , the filament length. **a**, Flapping frequency; **b**, amplitude. The disturbance level needed for the jump from a steady state to the flapping state decreases with increased filament length, up to a limit where a steady state can no longer survive.

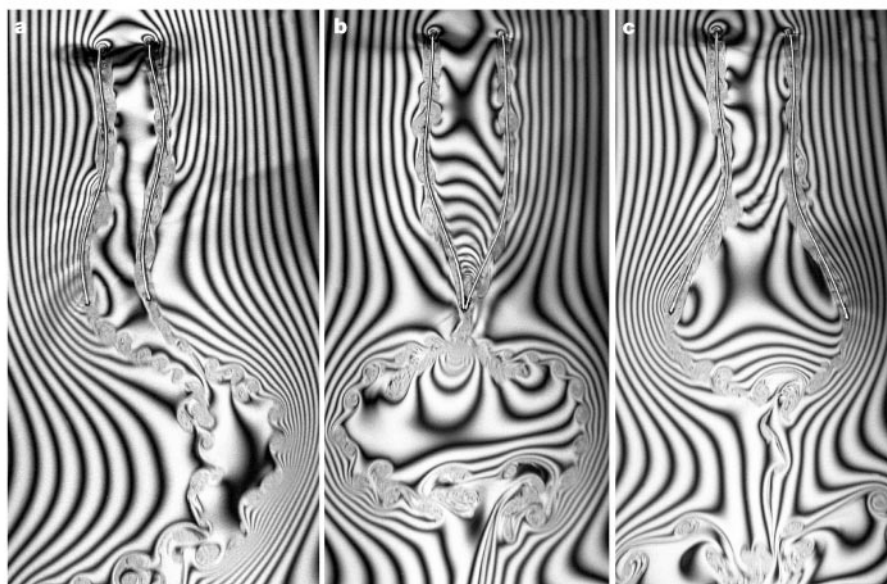


Figure 4 Dual filaments of the same length oscillating at the same frequency, when $L = 3.5$ cm and $U = 220$ cm s⁻¹. **a**, In-phase oscillation, with $d/L = 0.17$; **b** and **c**, out-of-phase oscillations, with $d/L = 0.25$. When two filaments touch, the local instantaneous thickness of the soap film is expected to increase to a dimension

comparable to the filament diameter. As opposed to the single-filament case, the soap film is evidently stretched and compressed, as is indicated at places where the interference fringes are almost perpendicular to the flow direction.

(Fig. 4a). As the distance is increased ($d/L > 0.21$), the filaments switch to become locked in an out-of-phase state (Fig. 4b and c) where they flap symmetrically about the centre-line. Stretching and compression of the film is evident from the interference patterns, where many fringes between the filaments are perpendicular to the flow direction. When the tips of both filaments touch each other, the film is significantly compressed. The number of fringes seen in Fig. 4c indicates that the film thickness has increased by at least $3.2\text{ }\mu\text{m}$. After this point of singularity, the enclosed fluid is released like a droplet and it spreads into the adjacent plane. The out-of-phase (symmetric) flapping state has an approximately 35% higher frequency than the in-phase state. As d is further increased, the coupling between the filaments weakens, and each tends to behave independently of the other. Moreover, filaments can be forced, from any of these states (at least over a modest range of lengths), into being stretched straight. This is the third cooperative state.

Historically, the flapping of sails and flags has interested fluid dynamicists primarily as an apparent indication of the growth of disturbances in a fluid flow. Rayleigh¹ relates the flag to the Kelvin–Helmholtz instability—the instability of an interface between two distinct, parallel streams—in the case where both streams have the same density and far-field velocity. Prandtl² discusses the dynamics of the interface as an instance of curvature effects on velocity. At any bulge in the flag the velocity is enhanced on one side and reduced on the other, the flapping being associated therefore with vorticity on the dividing streamline. From either viewpoint the disturbances are owing to vorticity in the neighbourhood of the flag. This vorticity, perhaps shed from an upstream flagpole, tends to be swept downstream with the flow, and its streamwise variations cause ripples in the flag that grow roughly linearly with distance downstream.

The phenomena reported here are not, however, caused by vorticity introduced at a flagpole. They seem instead to be associated with features mostly neglected in the classical descriptions—the filament's tension, elastic rigidity and mass, in addition to the dynamic interaction of the filament with the fluid flow. Indeed, near the transition point to flapping, the mass of the filament has come into balance with the mass of the interacting fluid, while the elastic energy of the filament balances the kinetic energy of the fluid. This is not so for shorter filaments, where the dynamical effect of the fluid flow is relatively lessened. We propose that this balance of effects induces a bifurcation to instability of the stretched-straight state, and leads to the flapping of the filament.

The suspended filament studied here is an open system with similarities to classical aerodynamic flutter (for example, see ref. 15), and to confined flows with compliant boundaries, studied for example in models of blood flow^{14,16}. The vorticity shed from the downstream edge introduces a fundamental difference between confined flows and the open finite filament, and the conditions determining the shed vorticity perhaps play an important role in our dynamic problem. The linear stability of a two-dimensional flexible flag has been recently studied theoretically¹⁷, though under strong simplifications, such as the use of a quasi-steady Bernoulli equation that, among other things, neglects vortex shedding and wake vorticity. Whereas this work does predict a bifurcation to instability with increasing filament length, it occurs at much smaller lengths than are observed here.

There are also peculiarities of soap film flows that would not be present in a two-dimensional viscous flow, if such a system could be otherwise realized. For example, to film stretching and compression, a soap film can manifest Marangoni (short-time response) and Gibbs (long-time response) elasticity¹⁸. However, if present, we expect neither of these resistive responses to provoke an instability, as is apparently observed in the transition from the stretched-straight state. Instead, these elastic responses should help set flapping frequencies and wavelengths, in coordination with the filament stiffness. Moreover, an examination of the interference patterns in the single-filament case shows little evidence of

dynamical compression (or stretching) of the film, because the flow is accelerating in those areas where compression is expected. Conversely, film compression and expansion is more evident in the two-filament case (compare Fig. 4b and c), although the detailed effect is as yet unknown.

We observe that the cooperative in- and out-of-phase states in the interacting filament experiments are consistent with the theory for a pair of coupled oscillators considered as two identical “vortex emitters”¹⁹. There, the strength of the coupling combined with the nonlinearity of the system causes oscillations in two modes with different symmetries. In an earlier work^{20,21}, the coupling of vortex streets shed from short cylinders were studied experimentally and different symmetries of coupled wakes were observed. In this case, the flow obstacles were solid and immobile.

Swimming offers alternatives comparable to the bistability of our filament. The stretched-straight state is the analogue of a glide, whereas the flapping state is analogous to swimming. Efficient propulsion uses the natural oscillations of the swimmer, which in the filament is a property mediated by stiffness. □

Methods

Set-up of the flowing soap film

Two thin nylon wires (0.3 mm in diameter) separate at a nozzle (0.5 mm inner diameter) attached to the bottom of a reservoir, which contains soapy water maintained at a fixed pressure head. A stopcock regulates the flow rate through the nozzle. The wires extend downwards to a lower container 2.4 m below. The spreading angle of the two wires at the top is about 8.3 degrees. From the interference pattern, the film thickness is seen to vary smoothly across the film by about 20% from its mean. Owing to air drag, a terminal velocity is nearly reached within approximately 60 cm from the nozzle⁶. In air, the velocity profile near the centre is close to being uniform, with velocity differences within 5% of mean, over 65% of the span about the midline. The filament is fixed using a thin tube (0.6 mm in diameter) perpendicular to the soap film. The Reynolds number for the filament is $Re = UL/\nu$, where U is the upstream flow speed, L the length of the filament and $\nu \approx 0.04\text{ cm}^2\text{ s}^{-1}$ the kinematic viscosity of the soap film⁵.

Velocity measurement

The soap solution is seeded with small polystyrene beads, $2\text{ }\mu\text{m}$ in diameter. A charge-coupled device (CCD) camera records the migration of the beads from frame to frame, when the flow is illuminated with a stroboscope operating at twice the video frequency. Velocity is determined by measuring of displacement of beads from any two successive frames. The accuracy is better than 0.4%. For velocity time series, a laser Doppler velocimeter (Model LDP-100, TSI Inc.) was used.

Flow and filament visualization

The flow is visualized using an interference technique, with a low-pressure sodium lamp as the monochromatic light (589.0 nm and 589.6 nm) illuminating the soap film. Two water–air interfaces of the film render an interference pattern, whose fringes show thickness contours of the film. The thickness variation from bright to bright (or dark to dark) fringes is $0.22\text{ }\mu\text{m}$. Because the thickness of the film is advected as a passive scalar by the flow field³, the interference pattern captures the flow pattern qualitatively.

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Observation of five-fold local symmetry in liquid lead

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The local point symmetry of the short-range order in simple monatomic liquids remains a fundamental open question in condensed-matter science. For more than 40 years it has been conjectured^{1–4} that liquids with centrosymmetric interactions may be composed of icosahedral building blocks. But these proposed mobile, randomly orientated structures have remained experimentally inaccessible owing to the unavoidable averaging involved in scattering experiments, which can therefore determine only the isotropic radial distribution function. Here we overcome this limitation by capturing liquid fragments at a solid–liquid interface, and observing the scattering of totally internally reflected (evanescent) X-rays, which are sensitive only to the liquid structure at the interface. Using this method, we observe five-fold local symmetry in liquid lead adjacent to a silicon wall, and obtain an experimental portrait of the icosahedral fragments that are predicted to occur in all close-packed monatomic liquids. By shedding new light on local bond order in disordered structures such as liquids and glasses, these results should lead to a better microscopic understanding of melting, freezing and supercooling.

The crystalline solid state is characterized by a long-range positional order of the atomic density at position $\mathbf{r}$, $\rho_s(\mathbf{r}) = \sum_m \delta(\mathbf{r} - \mathbf{R}_m)$, where $\mathbf{R}_m$ denotes the lattice positions of the atoms, and an associated orientational bond order belonging to one of the well-known allowed one-, two-, three-, four- or six-fold crystalline symmetries. In a diffraction experiment, δ -function Bragg reflections

$$S_s(\mathbf{q}) = \int \rho_s(\mathbf{r}) e^{i\mathbf{q}\cdot\mathbf{r}} d\mathbf{r} = \sum_{HKL} \delta(\mathbf{q} - \mathbf{G}_{HKL}) \quad (1)$$

that is, ‘Laue spots’, are observed that show the associated point symmetry of the lattice. Here $\mathbf{q} = \mathbf{k}_f - \mathbf{k}_i$ is the momentum transfer

given by the incident and diffracted wavevectors $\mathbf{k}_i$ and $\mathbf{k}_f$, respectively, and $\mathbf{G}_{HKL}$ are the reciprocal lattice vectors). In contrast, the liquid state exhibits short-range order extending only a few atomic distances given by the liquid correlation length ξ , which is typically in the range 3–8 Å in simple liquids. It can in principle be determined from the associated liquid pair correlation function $g(r)$. Even simple liquids can be considerably supercooled⁵, indicating a pronounced barrier to the transition between the supercooled metastable liquid and the thermodynamically stable solid. This supercooling phenomenon may be understood by assuming that the local liquid correlations have non-crystalline symmetries that have to be broken in the crystallization process. In fact, since the early work by Frank¹, Bernal^{2,4} and Scott³, a number of authors have proposed that the local liquid correlations in monatomic liquids, which exhibit centrosymmetric interactions favouring a close-packed local environment, are composed of defective or fragmented icosahedral building blocks that exhibit a five-fold symmetry^{1–4,6–8} (Fig. 1a). As these local liquid correlations are isotropically distributed, a conventional diffraction experiment unavoidably yields a 4π average:

$$S_l(q) = 1 + \rho_o \int [g_l(r) - 1] e^{i\mathbf{q}\cdot\mathbf{r}} d\mathbf{r} \quad (2)$$

this permits access only to the pair correlation function $g_l(r)$ of the liquid. Consequently, the intrinsic local liquid symmetry has withstood any experimental access. For a scattering experiment attempting to reveal the local liquid symmetry, a microscopic mechanism has to be provided that is able to capture the liquid building blocks in space and time. Here we show that this can in fact be achieved at solid–liquid interfaces by an appropriate combination of solid and liquid components.

At solid–liquid interfaces, the crystalline potential acts on the adjacent liquid in two ways. (1) Perpendicular to the wall, a liquid density oscillation appears within an interfacial layer of thickness ξ^* (see ref. 9 for a review). This hard-wall-induced packing effect has been observed at various solid–liquid interfaces^{10–12} as well as at free metallic liquid surfaces^{13,14}. (2) In the lateral direction $\mathbf{r}_\parallel$, the

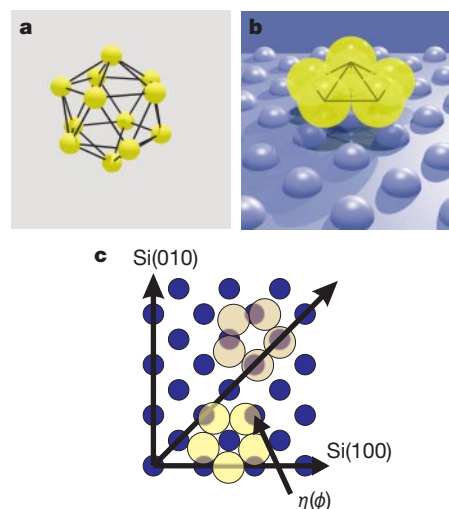


Figure 1 View of the dominant motif in the structure of bulk liquid lead and of interfacial liquid lead. **a**, Polytetrahedral arrangement predicted for close-packed monatomic liquids. **b**, Upper (pentagonal) half of the Pb icosahedron (**a**) captured by the potential landscape of the primitive Si(001) surface. **c**, Projection of the pentagonal structure onto four-fold coordinated sites of the Si(001) surface (lower pentagon, upper site position with minimum overlap of the projected electron density for rotation angle $\phi_n = 2\pi n/20$, where n is an integer; upper pentagon, hollow site with minimum overlap for $\phi_n = 2\pi(n+1/2)/20$; the overlap is denoted by η and shown in Fig. 3c as a function of the rotation ϕ of the pentagon).

† Deceased.

periodic wall potential $V(\mathbf{r}_\parallel) = \sum_{HK} V_{HK} \exp(iq_{HK}\mathbf{r}_\parallel)$ imposes its periodicity, as reflected in the coefficients V_{HK} (ref. 15), onto the 'contact-liquid', where H, K are conventional Miller indices.

Various theories have considered the case of a Lennard–Jones liquid that wets its own solid phase^{16–18}. Here we consider a situation in which the wall and the liquid consist of incompatible materials: a monatomic liquid metal in contact with a solid semiconductor. In this case, the wall exhibits only a small lateral periodic perturbation potential $V(\mathbf{r}_\parallel)$, which is incommensurate with the liquid nearest-neighbour distances. Consequently, there is no wall-induced long-range translational ordering. On the other hand, the substrate potential may still be strong enough to capture a fraction of the predicted bulk liquid building blocks in preferred orientations, giving rise to orientational alignment. A properly designed X-ray scattering experiment from these aligned liquid fragments should then allow access to their local symmetry. For the experiment described below, we have chosen the incommensurate combination of liquid lead—which exhibits close-packed face-centred-cubic, f.c.c., structure (lattice constant $a_{\text{Pb}} = 4.95 \text{ \AA}$ in the solid state)—in contact with Si(001), which has a diamond lattice with $a_{\text{Si}} = 5.43 \text{ \AA}$.

To date, no experimental information regarding the lateral microscopic structure of a liquid in contact with a wall has been available. This is due to the difficulties of designing a scattering experiment that is sensitive only to the liquid structure at the interface. It can, in principle, be achieved by exploiting total internal reflection of X-rays (Fig. 2a). In this scheme, a high-energy X-ray microbeam illuminates the interface from the solid side and is totally reflected. By this experimental device, non-propagating (evanescent) X-rays are created within the liquid adjacent to the wall while the X-ray field is absent in the bulk liquid. The in-plane liquid scattering from this evanescent X-ray field thus gives direct

access to the local liquid structure in contact with the wall. Such experiments are now just feasible at third-generation synchrotron sources. Figure 2b shows schematically such a synchrotron X-ray scattering experiment, as realized at the high-energy X-ray diffraction stations ID15A and BW5 of the European Synchrotron Radiation Facility (ESRF) and of the Hamburg Synchrotron Radiation Laboratory (HASYLAB), respectively^{19,20}.

We used two scattering schemes. (1) By using an in-plane detector scan (2θ scan, see Fig. 2b), we have measured the liquid structure factor parallel to the wall. A typical experimental result is shown in Fig. 3a, disclosing a scattering intensity distribution that is characteristic of bulk-like liquid correlations; this implies that the bulk liquid structure of Pb (ref. 21) is essentially preserved in the contact regime (see also ref. 22). In particular, the position of the first structure factor maximum, $q_1 = 2.18 \text{ \AA}^{-1}$, agrees with the known bulk value. (2) By maintaining the 2θ angle around the first diffraction maximum (see marked positions in Fig. 3a), azimuthal scans (ϕ scan, see Fig. 2b, c) were performed. In a bulk liquid, this type of scan inevitably shows a constant intensity associated with the random orientation of the liquid fragments. However, if the solid periodic potential is able to align preferred

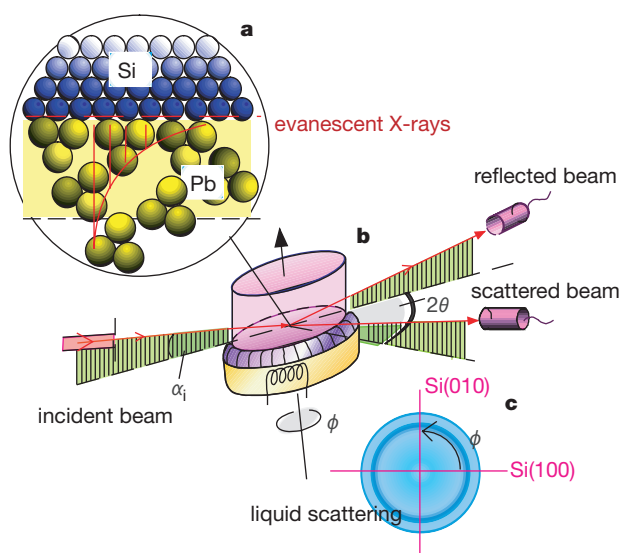


Figure 2 Schematic set-up of the diffraction experiment. **a**, A well defined synchrotron high-energy X-ray microbeam ($E = 71.5 \text{ keV}$, $8 \mu\text{m}$ size) penetrates the cylindrical Si solid ($r = 20 \text{ mm}$) from the side, and impinges at grazing angles on the Pb–Si interface. For incidence angles below the critical angle of total internal reflection ($\alpha_c = 0.041^\circ$) the incident beam is totally reflected. The angle of incidence α_i and the position of the interface in the X-ray beam are controlled to an accuracy of $4 \mu\text{rad}$ and $0.1 \mu\text{m}$. This allows the exclusion of any bulk liquid scattering contributions from the edges of the interface. **b**, In a specially designed ultrahigh vacuum (UHV) *in situ* diffraction chamber, the liquid Pb and the Si(001) surface are cleaned in UHV and then brought into contact at a temperature 10 K greater than the melting point of lead. A 360° Be window allows free access of the beam to the solid–liquid interface. **c**, Top view of the in-plane rotation ϕ at fixed momentum transfer q on the liquid scattering ring.

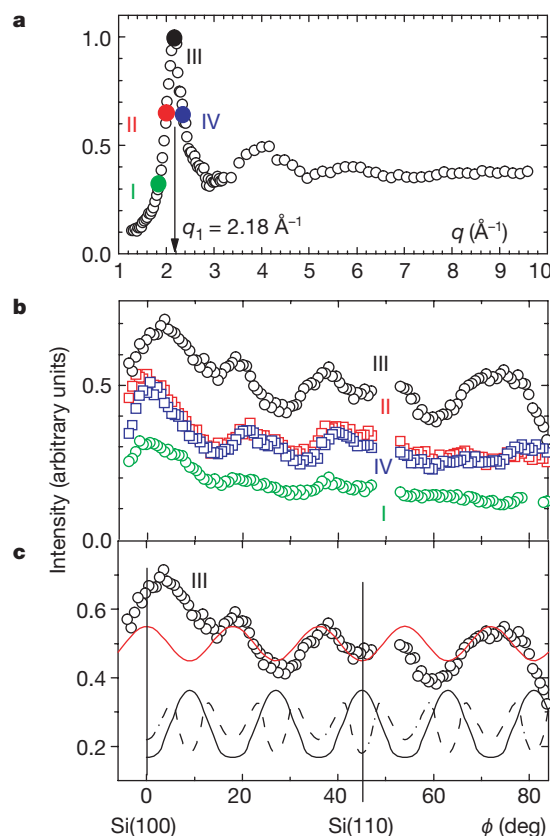


Figure 3 Scattering at the interface between liquid lead and Si(001). **a**, Structure factor of liquid Pb (2θ scan) measured in arbitrary units at an incidence angle $\alpha_i = 0.032^\circ$ well below the critical angle for total internal reflection. The evanescent X-ray wave 'tunnels' $55 \text{ \AA}$ deep into the liquid Pb (see Fig. 2a). The background scattering from the Si substrate has been measured and subtracted. The values of momentum transfer (I–IV) at which the in-plane anisotropy of the liquid structure factor has been tested experimentally are marked on the curve. **b**, Azimuthal distribution of the liquid scattering at the four fixed momentum transfer values (ϕ scans) marked in **a**. **c**, Azimuthal distribution of the liquid scattering in the first diffraction maximum. The red line is a fit of a sine wave to the experimental data in curve III (circles). The two lower lines show the total electron density overlap (see main text) of the pentagons with the underlying Si substrate versus pentagon rotation for the upper site position (full curve) and the hollow site position (dashed dotted curve). The epitaxial relation to the Si substrate is indicated by vertical lines.

orientations of the liquid building blocks, it should be possible to observe the characteristic intensity modulations from the local symmetry of these fragments. Our experimental observations for a ϕ scan within a 90° segment of the non-reconstructed four-fold Si(001) wall is shown in Fig. 3b: a pronounced modulation of the liquid scattering intensity is found. We observe five intensity maxima with a fixed epitaxial relationship to the substrate (Fig. 3b). We find this modulation only for momentum transfer values close to the maximum of the liquid structure factor (curves II–IV).

The non-crystalline symmetry of this intensity modulation can only be understood as a convolution of a four-fold and a five-fold symmetry, whereby the four-fold symmetry is clearly imposed by the Si(001) substrate and the orientational part of the associated Si–Pb interface interaction $V_o^{(\text{Pb-Pb})}$. This remains the only relevant interaction when Pb is adsorbed on Si(001) in the submonolayer regime. A similar situation has been investigated in detail by surface-sensitive X-ray diffraction at the Pb–Ge(111) interface²³, disclosing a substrate-modulated two-dimensional Pb liquid governed by the substrate symmetry only. This implies that the five-fold symmetry observed in our study emerges when bulk liquid Pb is brought in contact with the Si wall; that is, when the additional bulk Pb–Pb interaction $V_o^{(\text{Pb-Pb})}$ is introduced. The observed bulk-like in-plane liquid structure (Fig. 3a) gives unambiguous evidence that $V_o^{(\text{Pb-Pb})}$ is now dominating, while $V_o^{(\text{Pb-Si})}$ is a two-dimensional perturbation leading to a preferred alignment of a certain fraction $n_{\text{align}}(z)$ of the liquid building blocks.

The observed relative intensity modulation $\Delta I_{\text{mod}}/I$ in Fig. 3b, c is directly related to $n_{\text{align}}(z)$. To quantify this, if we denote ξ_{pb} as the correlation length within liquid Pb, $\xi_{\text{pb}} = 5\text{--}8\text{ \AA}$, then the depth-dependent alignment potential is given by

$$V_o^{(\text{Pb-Si})}(z) = V_o^{(\text{Pb-Si})} e^{-z/\xi_{\text{pb}}} \quad (3)$$

and in turn:

$$n_{\text{align}}(z) = 1 - e^{-V_o^{(\text{Pb-Si})}(z)/k_B T} \quad (4)$$

As the evanescent X-rays decay exponentially with depth within $\Lambda = 55\text{ \AA}$, $\Delta I_{\text{mod}}/I$ and $n_{\text{align}}(z)$ are related by²⁴:

$$\frac{\Delta I_{\text{mod}}}{I} = \frac{1}{\Lambda} \int_0^\infty n_{\text{align}}(z) e^{-z/\Lambda} dz \quad (5)$$

Inserting equations (3) and (4) into equation (5), and using the experimentally determined value $\Delta I_{\text{mod}}/I = 0.10 \pm 0.05$ (from Fig. 3c), the value of $V_o^{(\text{Pb-Si})}$ can be estimated numerically. This is shown in Fig. 4, which gives $V_o^{(\text{Pb-Si})} = 50\text{--}90\text{ meV}$ for the orientational part of the interface potential, depending on the detailed assumption of the interface-related value of ξ_{pb} (which may be

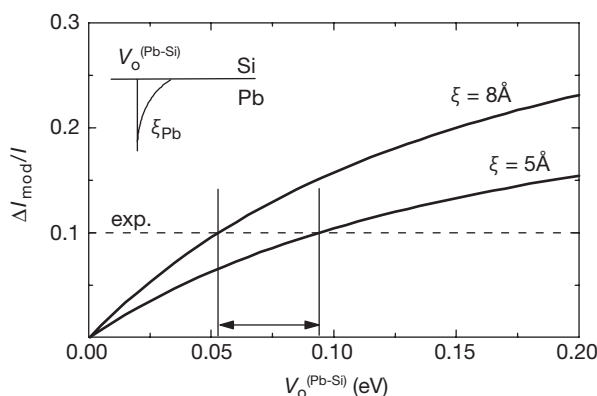


Figure 4 Determination of the Si–Pb interface potential from the amplitude of the azimuthal relative intensity modulation $\Delta I_{\text{mod}}/I$. Assuming a range of $5\text{--}8\text{ \AA}$ for the specific value of the bulk correlation length ξ_{pb} (see inset) we obtain a range of $50\text{--}90\text{ meV}$ for the interface potential.

slightly different from the bulk value). Thus, the observed value of the modulation amplitude is directly correlated with a thermal interface energy, and comparable to $kT_m \approx 50\text{ meV}$ at the melting temperature (T_m) of lead, 600.6 K . The value of the bulk binding energy $V_o^{(\text{Pb-Pb})}$, on the other hand, is given by the cohesive energy $V_o^{(\text{Pb-Pb})} = 2.0\text{ eV}$, implying that $V_o^{(\text{Pb-Si})}/V_o^{(\text{Pb-Pb})} \approx 10^{-2}$.

Figure 1b shows such liquid building blocks (the upper fragment of the icosahedron of Fig. 1a) attached to the Si(001) surface such that one Si surface atom is located at the centre of a pentagon of Pb atoms, thereby giving rise to the four-fold symmetric repetition of the five-fold modulation. The energetically favourable in-plane orientations of these pentagons are then found by determining those orientations (see Fig. 1c) where the projected electron density of the pentagonal building blocks has the minimum overlap (η in Fig. 1c) with the underlying Si atoms. This geometric argument predicts the maxima of the measured azimuthal intensity distribution at the experimentally observed positions (see Fig. 3c). The overlap varies over the underlying Si atoms, and this qualitatively accounts for the breadth of the five-fold oscillations. Also shown (in Fig. 1c) is a simulation for Pb pentagons centred at the other available four-fold coordinated site on the primitive Si(001) surface, the hollow site (Fig. 1c). This adsorption site is excluded unambiguously by our data. $\square$

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Role of sea surface temperature and soil-moisture feedback in the 1998 Oklahoma–Texas drought

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The drought that affected the US states of Oklahoma and Texas in the summer of 1998 was strong and persistent, with soil moisture reaching levels comparable to those of the 1930s ‘dust bowl’^{1,2}. Although other effects of the record-strength 1997–98 El Niño were successfully predicted over much of the United States, the Oklahoma–Texas drought was not³. Whereas the response of the tropical atmosphere to strong anomalies in sea surface temperature is quite predictable, the response of the extratropical atmosphere is more variable^{4,5}. Here we present results from mechanistic experiments to clarify the origin and maintenance of this extratropical climate extreme. In addition to global atmospheric models^{6–11}, we use a regional model^{12,13} to isolate regional climate feedbacks. We conclude that during April and May 1998, sea surface temperature anomalies combined with a favourable atmospheric circulation to establish the drought. In June–August, the regional positive feedback associated with lower evaporation and precipitation contributed substantially to the maintenance of the drought. The drought ended in the autumn, when stronger large-scale weather systems were able to penetrate the region and overwhelm the soil-moisture feedback. Our results show the potential for numerical models including appropriate physical processes to make skilful predictions of regional climate.

The strong Oklahoma–Texas drought had already been established by May 1998, with high temperatures and a low-precipitation weather pattern typical of July and August. It persisted until early October throughout most of Oklahoma and Texas, except in southern Texas, where it rained in August and September. The drought was not predicted by the US operational seasonal forecasts made in March 1998³ at the National Centers for Environmental Prediction

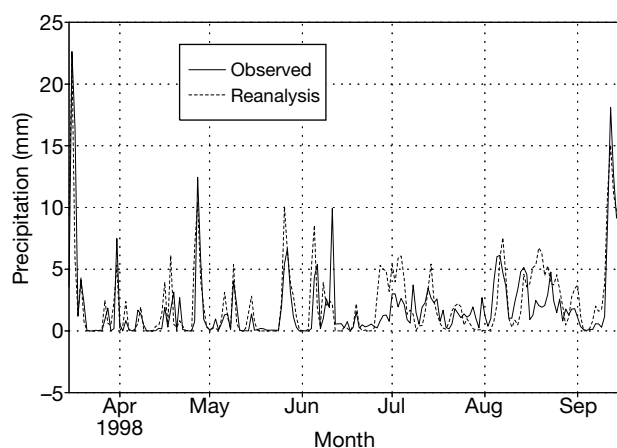


Figure 1 Comparison of observed precipitation and that predicted by the NCEP/NCAR reanalysis. The precipitation is averaged for the Oklahoma–Texas area (29° N–37° N, 105° W–91° W) during 1998.

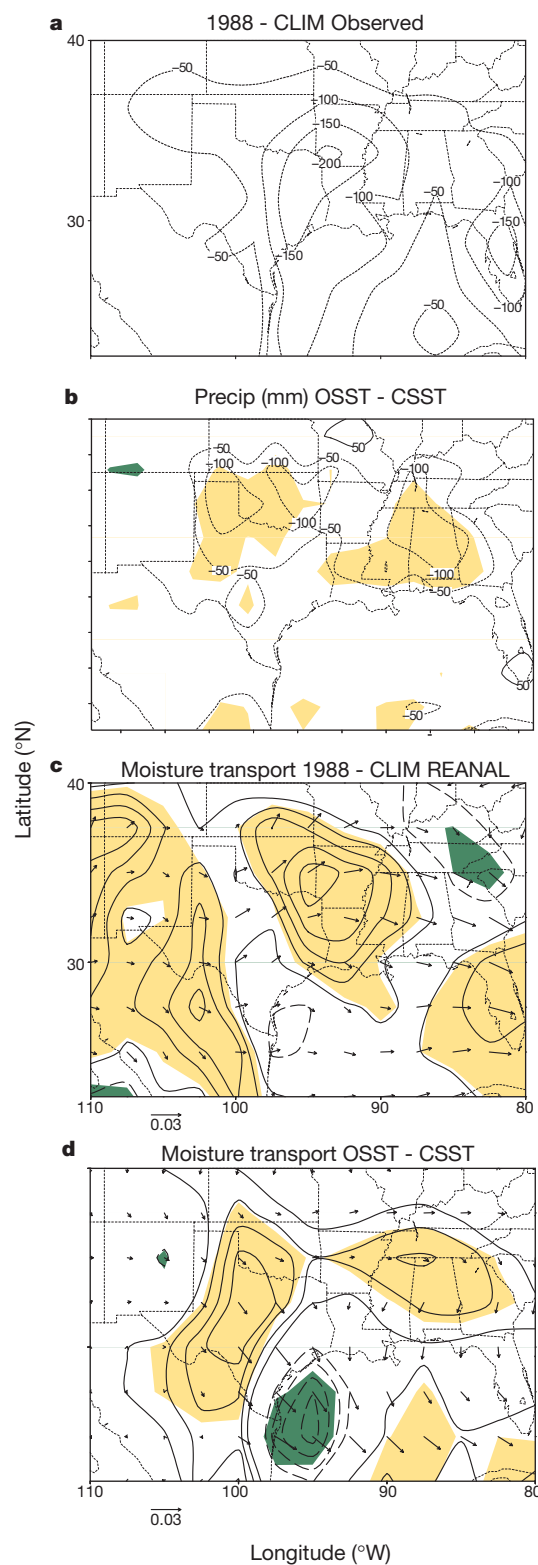


Figure 2 Comparison of the model impact of SST anomalies (OSST – CSST) with the observed anomalies for May 1998. **a**, Observed precipitation anomalies. **b**, Difference between the OSST and CSST precipitation. **c**, Moisture transport (arrows) and moisture divergence (contours) averaged for low levels (925 hPa and 850 hPa), anomalies computed from the reanalysis. **d**, As **c**, but for the OSST – CSST experiments. Contour intervals for moisture divergence are $0.5 \times 10^{-8} \text{ kg kg}^{-1} \text{ s}^{-1}$, without zero line. Shading indicates differences statistically significant at the 95% level, with yellow (green) for increased (decreased) divergence. The scale of the moisture transport vector ($\text{kg kg}^{-1} \text{ m s}^{-1}$) is indicated below panels **c** and **d**.

(NCEP). It is important to establish the mechanisms that originated, maintained and ended the drought in order to establish whether such extreme drought is predictable.

There are three main mechanisms that can produce extended atmospheric anomalies: (1) sea surface temperature (SST) anomalies, (2) soil-moisture anomalies, and (3) atmospheric initial conditions favourable to such a climate extreme even in the absence of surface forcing (that is, internal forcing). Tropical SST anomalies, such as those associated with El Niño and La Niña episodes, have a profound effect in tropical and subtropical regions⁴. A drought can also be enhanced through a regional positive feedback: dry soil reduces local evaporation and therefore can reduce precipitation^{6,7,10,11}. It is also possible that the atmosphere in the late spring of 1998 was already 'primed' to produce a drought in the Oklahoma–Texas region even without anomalies of SST or soil moisture⁸.

It would seem natural to "blame El Niño" of 1997/1998 for the drought, but there are problems with this simple assumption. First, warm El Niño/Southern Oscillation (ENSO) episodes tend to be correlated with high precipitation in April–June over Oklahoma and Texas^{14–16}. In particular, the drought of 1988 in the Great Plains has been attributed to La Niña (cold) conditions^{6,9,17}. Furthermore, the warm ENSO episode faded in June 1998, and was quickly replaced with strong La Niña (cold SST) conditions in the equatorial Pacific Ocean. Conversely, soil-moisture anomalies alone could not have triggered the drought, because the Oklahoma–Texas region had above-normal precipitation during the early spring, so that the soil was wetter than normal in the spring of 1998.

For this reason, we tested alternative hypotheses, using numerical models and mechanistic experiments. The hypotheses were: (1) both atmospheric conditions in the spring and the SST anomalies

associated with the waning warm ENSO episode were crucial in the establishment of the anomalous Oklahoma–Texas drought conditions during the spring of 1998. (2) The consequent deficits in the soil moisture contributed to the maintenance of the drought throughout the summer and early autumn through a local positive feedback between the atmosphere and the land. (3) The drought ended in the autumn, when stronger weather systems were able to penetrate this region and overwhelm the soil-moisture feedback.

Tropical SST anomalies have a global-scale effect, whereas soil-moisture anomalies have a much more regional effect. Because the two mechanisms invoked for the drought during the spring and summer are quite different, we need different modelling approaches to test the first two hypotheses. To test the first hypothesis we integrated a version of the NCEP global spectral model (GSM)¹⁸ with climatological and observed SSTs, and with 1998 and 1993 initial conditions. For the second and third hypotheses, based on regional feedbacks, we needed to maintain the observed global circulation, so we opted for running the NCEP regional spectral model (RSM)^{12,13}. The horizontal resolution of the GSM and RSM is about 200 km and 50 km, respectively. We first checked whether the NCEP/NCAR 50-year reanalysis¹⁹ anomalies in precipitation were reasonable, as they determine the soil-moisture anomalies. Figure 1 compares the observed and the reanalysis precipitation centred over the Oklahoma–Texas region (which we define as the region bounded by 29°N–37°N and 105°W–91°W) from mid-March to mid-September 1998. It shows that the reanalysis 6-hour forecast reproduced very well the amount and timing of the observed precipitation. Both this, and other comparisons with surface and soil observations from the Oklahoma Mesonet¹ of surface observations, provide confidence that the reanalysis can indeed be used for this purpose.

To check the influence of the SST anomalies, we performed global

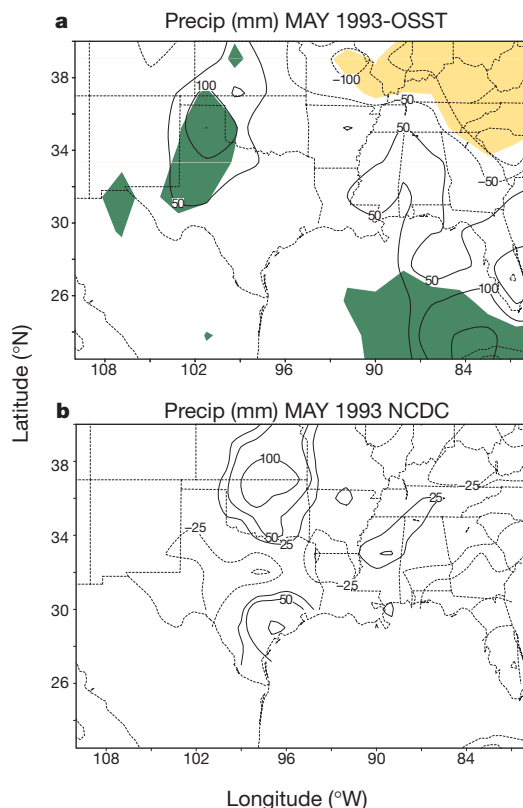


Figure 3 Effect of the initial conditions on the drought. **a**, Precipitation difference between the '1993' and OSST experiments, and **b**, the observed precipitation anomalies in May 1993. Shading in **a** indicates differences statistically significant at the 90% level (green for increased and yellow for reduced precipitation).

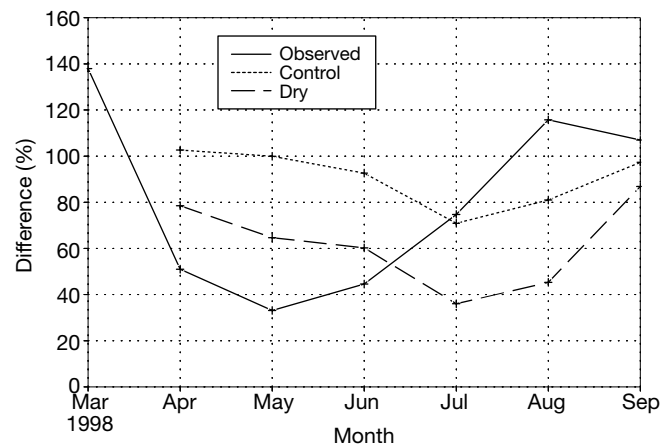


Figure 4 Effect of the soil-moisture feedback on the drought. The figure shows the percentage difference in precipitation over the Oklahoma–Texas region. Solid lines, observed minus climatology; short-dashed lines, RSM CONTROL minus CLIMAT; long-dashed lines, DRY minus CLIMAT.

Table 1 Percentage change of monthly precipitation

	April	May	June	July	August	September
OBS	–48	–65	–55	–25	15	8
SST	–31	–40	–7	NA	NA	NA
Soil moisture	2	0	–8	–30	–18	–2
Internal forcing	(–19)	–29	(–40)	NA	NA	NA

Percentage change of monthly precipitation (%) over the Oklahoma–Texas region due to the contribution of SST and soil-moisture anomalies estimated from the global (OSST–CSST) and regional (CONTROL–CLIMAT) models, respectively. The reduction of observed precipitation (OBS) relative to the climatology during 1949–98 is also shown. NA indicates that estimates from experiments are not available. Internal forcing for May 1998 represents the effect of the initial conditions for May 1998 versus the '1993' initial condition experiments. Values in parenthesis are estimates of internal forcing that would be required to explain the observed deficits.

ensembles of five forecasts starting from initial conditions corresponding to the 5 days of 26–30 April 1998, and verified during the month of May 1998. Two experiments were run: OSST, with observed SST in 1998; CSST, with climatological SST. To test the effect of the initial conditions an additional ensemble experiment denoted '1993' was run with SST and soil moisture anomalies for 1998, as in OSST, but with initial conditions corresponding to 1993 (a flood year).

Figure 2a shows the observed deficit in precipitation for May 1998, and Fig. 2c shows anomalous low-level moisture transport from the Gulf of Mexico into the Oklahoma–Texas region. They suggest that the drought in May was associated with a lower than normal input of moisture into the region from the low-level jet coming from the Gulf^{20,21}. Figure 2b and d show the effect of the SST anomalies present during May. They indicate that the SST anomalies contributed to the establishment of the drought by producing a significant reduction in precipitation, enough to explain about 60% of the deficit observed at that time. Similar experiments were performed for April and June 1998 and the results, summarized in Table 1, generally confirm those obtained for May 1998.

The extent to which the atmospheric conditions in 1998 were already 'primed' to generate the drought is shown in Fig. 3. It is clear that in 1998 the atmospheric circulation would have resulted in lower precipitation than in 1993, even if the surface forcing from SST and soil moisture were the same in both years. The reduction is about 40% of the observed deficit (Table 1).

We tested the second hypothesis (maintenance of the drought through soil-moisture positive feedback) using the regional spectral model. Reanalysis boundary conditions and SSTs were used to drive the RSM during April–September 1998. Only the bottom (deep) soil-moisture model layer was updated from the reanalysis every 24 hours during the model integration period, while the soil moisture in the top (shallow) layer was predicted, to maintain balance with the atmospheric forcing.

Three types of runs were made with the regional spectral model. The first two, CONTROL and CLIMAT, had deep soil moisture replaced by the reanalysis soil moisture for 1998 and the reanalysis 50-year climatology, respectively. As these RSM experiments changed only the lowest soil model layer, they probably underestimated the positive feedback on the drought. To test the effect of a more intense deficit in the soil moisture, we also performed an experiment (DRY) in which the lower-soil model moisture was maintained at the wilting point. The results are presented in Fig. 4. They suggest that soil-moisture anomalies alone would have in fact resulted in an increase in precipitation during April and May, as the early spring had above-normal precipitation and the soil was wetter than normal at the beginning of this period. However, during the summer, soil-moisture feedback could maintain an extreme drought through local feedback. The fact that by September the precipitation recovers to near CLIMAT levels even in the DRY experiment, supports the third hypothesis—that the end of the drought in the autumn was due to stronger events overwhelming the regional positive feedback.

We introduced in this study the use of a regional model nested within the observed large-scale forcing in order to isolate the local feedback mechanism between soil moisture and precipitation. The results suggest that this extratropical climate extreme is not simply rooted in a tropical SST forcing and/or soil-moisture anomalies^{2,3,6,7,9,17}. Rather, it is due to a nonlinear coupling of SST anomalies and atmospheric internal forcing organized in the previous months, and a physical interaction with soil-moisture anomalies. At present, statistical seasonal climate predictions are still more skilful than predictions of numerical models²². Our results suggest that research with dynamic models, including appropriate physical processes and improved initial conditions, have the potential to overcome this disadvantage. □

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Possible presence of high-pressure ice in cold subducting slabs

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During the subduction of oceanic lithosphere, water is liberated from minerals by progressive dehydration reactions^{1,2} and is thought to be critical to several geologically important processes such as island-arc volcanism³, intermediate-depth seismicity⁴ and chemical exchange between the subducting lithosphere and mantle⁵. Although dehydration reactions would yield supercritical fluid water in most slabs, we report here that the stable phase of H₂O should be solid ice VII in portions of the coldest slabs. The formation of ice VII as a dehydration product would affect the generation, storage, transport and release of water in cold sub-

duction zones and equilibrium conditions of dehydration would shift, potentially affecting the depths of seismogenesis and magmagenesis. Large amounts of pure ice VII might accumulate during subduction and, as a sinking slab warms, eventual melting of the ice would release large amounts of water in a small region over a short period of time, with a significant positive volume change. Moreover, the decreasing availability of fluid water, owing to the accumulation of ice VII and its subsequent reaction products in a cooling planetary interior (for example, in Mars or the future Earth), might eventually lead to a decline in tectonic activity or its complete cessation.

Supercritical fluid water is the stable phase of H_2O at high pressure and temperature, but at low temperature the stable high-pressure phase is the cubic (space group $Pn3m$) solid ice VII^{6,7}. We investigated whether conditions within some subducting lithospheric slabs fall within the ice VII stability field. The recently measured melting curve of ice VII is shown in Fig. 1. Superimposed on this curve are a range of subduction zone thermal models, giving minimum temperatures inside model slabs, together with a mean mantle geotherm. We note that, given the uncertainties in slab thermal models (see Methods), the temperature curves for the cooler families of slabs cross the phase boundary between water and ice VII.

Also shown in Fig. 1 are equilibrium reaction boundaries for three representative dehydration reactions—brucite to periclase⁸, lawsonite blueschist to quartz eclogite^{9,10} and antigorite (serpentine) to enstatite plus the dense hydrous magnesium silicate 'phase A'². The latter two reactions are believed to be important in terrestrial subduction zones^{11,12}. These phase relations for melting and dehydration will also be sensitive to the solubility of solid phases in the aqueous phase. What are the potential effects of forming solid ice VII instead of supercritical fluid water by dehydration during subduction?

One effect of initial dehydration in the stability field of ice VII rather than of water is to change the sign of the Clapeyron slope (dP/dT) of the dehydration reaction, because of the low entropy of ice VII relative to that of water. Thus, the onset of dehydration will be at lower pressures, and hence at shallower depths, in the ice VII stability field. The pressure–temperature (P – T) dehydration curves will be more nearly linear (constant $\Delta S/\Delta V$) when ice rather than fluid is formed, because of the expected smaller

compressibility and thermal expansivity of ice. Thus the dehydration reaction will resemble a solid–solid phase transition. The volume change (ΔV) of dehydration becomes significantly more negative (by $\sim 50\%$) at the onset of ice VII formation; hence a greater reduction in free energy is available for a given pressure overstep of the reaction boundary. This increases the driving force of reaction and may inhibit metastable persistence of the hydrated phase assemblage. On the other hand, the absence of a fluid phase in which the minerals can dissolve may hinder the kinetics of dehydration, which might otherwise occur by a dissolution–reprecipitation mechanism. Moreover, these changes in dP/dT and ΔV reflect a change in sign of the enthalpy of reaction (ΔH), so that the endothermic dehydration reactions become exothermic upon entering the ice VII stability field. Thus the heat balance, driving force, and rates of dehydration are expected to be different when the evolved H_2O is solid.

The release of H_2O as ice VII from hydrous minerals allows its continued transport downward in solid form, rather than its rapid lateral and upward movement as a fluid. The lower mobility and buoyancy of ice VII implies less rapid infiltration and ascent of H_2O , allowing the possibility of later water-consuming reactions, such as the incorporation of H_2O into wadsleyite ($\beta\text{-Mg}_2\text{SiO}_4$)¹³ or the formation of dense hydrous magnesium silicates such as phase A'¹⁴, if the H_2O released as ice VII continues moving downward with the slab. Furthermore, the absence of fluid water as a solvent implies minimal transport of solutes, such as incompatible elements, as long as H_2O remains crystalline. Thus the H_2O formed as ice VII will not carry the geochemical signature of its initial formation conditions. However, as soon as this ice melts, it will form a corrosive fluid, pure water, which will react rapidly with the rocks where it first forms. The resulting mineral–water equilibrium and geochemical signature will represent the greater depth at which ice VII melted, rather than the range of shallower depths over which dehydration occurred.

The formation of ice VII from hydrous phases will occur gradually^{11,14}, reflecting multivariant dehydration curves of multi-component mineral solid solution assemblages, but its later melting to water should be univariant (that of a pure single-component substance), occurring at one specific pressure and temperature as the P – T boundary is crossed. Furthermore, melting of ice VII should be much more rapid than the reconstructive dehydration

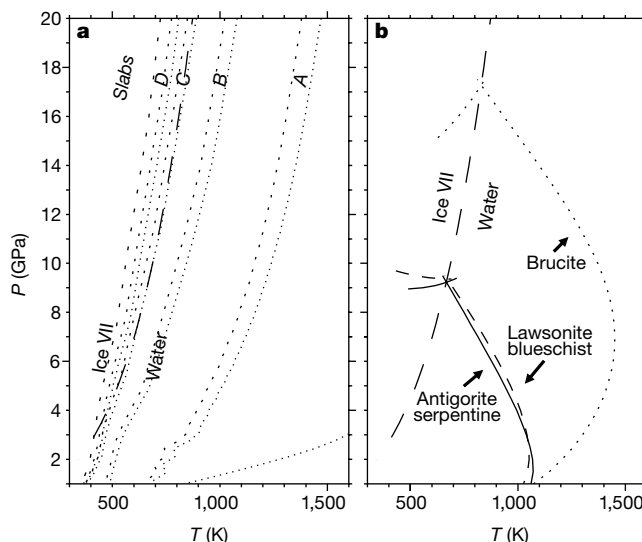


Figure 1 The melting curve of ice VII. Data from experimental measurements of melting conditions²⁸. Heavy dashed line shows the melting curve. **a**, Superimposed are the minimum temperatures inside slabs, calculated for a range of subduction zone thermal models (slab classes A, B, C and D) along with a typical mantle geotherm (dotted lines).

b, Superimposed are calculated equilibrium reaction boundaries for three representative dehydration reactions: brucite to periclase (dotted lines), lawsonite blueschist to quartz eclogite (dashed lines), and antigorite (serpentine) to enstatite plus phase A (solid lines).

of silicate minerals. This implies a sudden release of fluid H_2O when the slab leaves the ice VII stability field. Such sudden, rather than gradual, release of water has important implications for arc volcanism and seismicity. As both arc magmatogenesis and intermediate-depth seismogenesis are believed to be H_2O -mediated processes, the depth-distribution of melting and of earthquakes may reflect the availability of water as a function of depth along individual slab geotherms. To estimate the magnitude of these effects, if the hydrated portion of the slab contains about 6 wt% H_2O ¹⁴, then about 0.01 mole of H_2O can be liberated per cm^3 of material, corresponding to a volume change of about 1%. The approximately 0.1 cm^3 of water per cm^3 of material thus suddenly liberated is immediately available for melting, the transport of ions and other reactive chemistry.

The presence of solid H_2O within a slab should also perturb other subduction zone properties, particularly if this ice is carried downward until it melts or reacts chemically. Changes in buoyancy and rheology, for example, should arise from the low density and strength, respectively, of ice near its melting point. Aggregate seismic velocities, too, should be affected by the relatively low acoustic velocities in ice VII^{15,16}. Although it is perhaps mitigated by the high symmetry of cubic ice VII, the potential for growth of large ice crystals also raises the possibility of observable changes in elastic anisotropy.

The detection of ice VII under conditions of cold subduction is possible by *in situ* (infrared, Raman, synchrotron X-ray) experimental study, as is the potential for transport of H_2O to even greater depths by other higher-pressure ice polymorphs¹⁷. Quenched metastable ice VII can also be studied (by neutron diffraction) at ambient pressures¹⁸.

Evidence for the geophysical signature of ice VII could be sought, for example, in Tonga, one of the world's coldest subduction zones¹⁹, which also exhibits unusual patterns of seismicity and volcanism. Whereas subduction zone seismicity generally declines with increasing depth, attaining a minimum at 300–400-km depth before rising again, seismicity beneath Tonga exhibits a more marked increase in seismic moment release near 400 km depth than do other subduction zones. If seismicity at and above such depths is related to water, as is commonly asserted^{4,19}, then perhaps significantly greater amounts of water become available near this depth beneath Tonga than elsewhere. Furthermore, slow seismic velocity anomalies extend to 400 km beneath the Tonga volcanic arc and the adjacent Lau back-arc basin, suggesting that partial melting related to the release of volatiles from the slab may occur near this depth²⁰. If water is transported by ice VII beneath Tonga, then the ice may melt near this depth, providing water in a concentrated dose. Indeed, although the thermal models in Fig. 1 show the lowest temperatures in the slab at any depth, actual isotherms will migrate into the slab's interior as the slab surface warms with increasing depth, so that P – T paths followed by individual particles will have shallower slopes than depicted. Given the uncertainties in both the Tonga slab geotherm (class D in Fig. 1) and the ice VII melting curve, ice VII formed by dehydration near 150 km, for example, should melt somewhere in the 300–800-km depth interval. Thus, melting near 13 GPa (400 km) is possible. Furthermore, this pressure is near that at which wadsleyite becomes stable, so any released H_2O could potentially affect the α – β phase transition in olivine and thus the properties of the 410-km seismic discontinuity¹.

Of course, the dehydration of real multicomponent mineral phases produces an aqueous fluid containing dissolved ionic species. These dissolved species affect both the equilibrium dehydration curves and the ice melting equilibria; both are depressed in temperature. Thus, the extent to which a given local geotherm enters the ice VII field may be modified from that presented in a simple calculation, but the balance between the formation of solid ice by dehydration and the formation of an aqueous fluid will be affected less strongly than either reaction (ice melting and ordinary

dehydration) alone. The arguments presented above, and especially those for cooler planetary regimes (see below) remain valid, although the exact locations of phase boundaries will be affected. Indeed, owing to the low temperatures and the large latent heat of fusion for ice VII, reducing the fluid activity from unity to 0.9 should result in a melting-point depression of order 1–10 K over the pressure range 0–20 GPa.

Finally, the role of ice phases in subduction should assume a growing importance as a planet cools through geologic time. As its average geotherm becomes cooler, it is reasonable to expect that, if plate tectonics or a similar process is occurring, a growing fraction of subducting slabs will contain regions of ice VII stability. Thus, the phenomena discussed above will grow more important as a planet cools and should be considered in models of future Earth evolution. Ice VII may be even more important in the martian interior. Although the temperature distribution within Mars is poorly constrained, the planet's smaller size lends it a much faster cooling rate²¹, so that the interior should be colder than Earth's²². Any past episodes of subduction should have efficiently cooled the martian interior and effectively transported surface water (by a thick hydrothermally altered crust) to the deep mantle²¹, whereas the fraction of subducting slabs in the stability field of ice VII should have been much greater than on Earth. As a planet cools so that its normal geotherm approaches the ice VII field, progressively less subducted water may be returned to the surface; it will instead be stored in ice and/or hydrous mineral phases in the slabs. With less water, volcanism will diminish, breaking the plate tectonic cycle. Hence, growing transport of H_2O to the deep interior as ice VII and subsequent storage in a reservoir of ice or hydrous mineral phases may have served to deprive Mars of its earlier surface water, to limit deep melting, and to end (or considerably slow) martian plate tectonics. It has been suggested that late Tharsis volcanism on Mars may have involved deep H_2O release attributable to later radioactive heating²¹. Although the magnitude of radioactive heating is even more poorly constrained for the interiors of other planets than it is for Earth's, such late release of water is consistent with either ice VII melting or dehydration upon local reheating.

Thus, water initially present in planetary oceans becomes trapped in sediments, subducted and immobilized in the deep interior. Accumulation of ice VII or its by-product hydrous phases may eventually lead to a tectonically dead planet with a dry surface. Planetary bodies and their satellites in solar and extrasolar planetary systems are strikingly diverse, and understanding the origins and consequences of such variability is a major goal of planetary science. We suggest that the state (fluid versus crystalline) of H_2O in planetary interiors can profoundly affect both dynamics and thermal balance, and that changes in the relative proportions of fluid and solid H_2O can alter the subsequent evolution of a planetary body. Perhaps the presence or absence of ice VII in planetary interiors, like that of ice I on planetary surfaces, is one of the important factors influencing the different possible paths of planetary evolution. □

Methods

Calculation of subduction zone thermal models yields a range of possible slab geotherms, depending upon the various assumptions and parameters employed. The purpose of our calculations is not to argue for one model in preference to another but simply to generate a suite of reasonable temperature profiles. If a significant number of these intersect the melting curve of ice VII, then the main hypothesis of this Letter is supported. We computed subduction zone thermal models using a standard kinematic finite-difference method²³ for four thermal classes¹⁹ of slabs. Class A represents young slabs (age is 12 Myr, rate is 6 cm yr^{-1} , dip is 30°; for example, Cascadia, Mexico, Nankai Trough), class B represents middle-aged slabs (50 Myr, 7 cm yr^{-1} , 45°; for example, Middle America, northern Chile, central Aleutians), class C represents old slabs (140 Myr, 8 cm yr^{-1} , 60°; for example, eastern Indonesian, Kuriles), and class D represents old and rapidly subducting slabs (140 Myr, 14 cm yr^{-1} , 60°; for example, Tonga). To illustrate a range of thermal profiles (Fig. 1), for each class of slab we first computed thermal structures for a plate model with a high (1,450 °C, ref. 24, fine dotted curves) basal lithospheric temperature, thereby reproducing the published models for these four thermal classes¹⁹. We then re-computed thermal structures for the same plate models but with a moderate (1,325 °C, ref.

25, medium dotted curves) basal lithospheric temperature. Finally, for the coldest class, D, we also computed a thermal structure for a halfspace model with a moderate (1,325 °C, ref. 25, heavy dotted curve) basal temperature. These simple models neglect both shear and radiogenic heating, effects which would raise slab temperatures. However, we also neglect additional effects which would lower slab temperatures. In northern Tonga, for example, rates can reach 24 cm yr⁻¹, ref. 26), thus preserving colder slab temperatures to great depths. Moreover, the fixed kinematic slab boundaries of our simple model do not allow for the thermal insulation effect of the viscous blanket that develops on a slab's upper surface in a mantle with temperature-dependent viscosity, an effect which further depresses slab temperatures at depth in more sophisticated models of dynamically evolving slabs²⁷.

We used the melting curve of ice VII as determined from contrasting indices of refraction by direct visual observation²⁸, rather than from earlier studies based on the disappearance of diffraction peaks that provided only lower bounds on melting points⁶. From this curve, we used thermodynamic equations of state for water⁸ and ice VII^{6,29} to calculate a best-fit specific heat function and enthalpy and entropy values for ice VII, obtaining parameters ($\Delta H = -290.3 \text{ kJ mol}^{-1}$, $S^0 = 41.46 \text{ J K}^{-1} \text{ mol}^{-1}$, $C_p = 59.91 \text{ J K}^{-1} \text{ mol}^{-1} + 2.904 \times 10^{-3} T - 1.509 \times 10^{-6} T^2$) in reasonable agreement with those derived from earlier melting curves⁶. We then used these thermodynamic parameters to calculate equilibrium reaction boundaries for three representative dehydration reactions (brucite to periclase⁸, lawsonite blueschist to quartz eclogite^{9,10}, and antigorite (serpentine) to enstatite plus the dense hydrous magnesium silicate 'phase A'²), in both the water and ice VII stability fields, using the equations of a state for antigorite², brucite³⁰, phase A³¹, and the set of minerals periclase, glaucophane, lawsonite, jadeite, diopside, pyrope, quartz and enstatite³².

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Analysis of an evolutionary species–area relationship

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Large islands typically have more species than comparable smaller islands. Ecological theories, the most influential being the equilibrium theory of island biogeography¹, explain the species–area relationship as the outcome of the effect of area on immigration and extinction rates. However, these theories do not apply to taxa on land masses, including continents and large islands, that generate most of their species *in situ*. In this case, species–area relationships should be driven by higher speciation rates in larger areas^{2–6}, a theory that has never been quantitatively tested. Here we show that *Anolis* lizards on Caribbean islands meet several expectations of the evolutionary theory. Within-island speciation exceeds immigration as a source of new species on all islands larger than 3,000 km², whereas speciation is rare on smaller islands. Above this threshold island size, the rate of species proliferation increases with island area, a process that results principally from the positive effects of area on speciation rate. Also as expected, the slope of the species–area relationship jumps sharply above the threshold. Although *Anolis* lizards have been present on large Caribbean islands for over 30 million years, there are indications that the current number of species still falls below the speciation–extinction equilibrium.

The 143 species of Caribbean island *Anolis* lizards are ideal for a test of evolutionary theories of diversity. The group has been there since at least the middle Oligocene and has radiated extensively within the archipelago^{7–9}. Current species richness on islands is related to area, and both immigration and speciation have contributed^{10,11}. We used a phylogeny for Caribbean *Anolis* species based on mitochondrial DNA⁹ to estimate the number of immigration and speciation events on islands. By comparing these quantities with island area, we can test three predictions that derive from an evolutionary theory of species–area relationships: (1) a threshold island size should exist, above which speciation surpasses immigration as a source of new species; (2) above the threshold size, recorded speciation events per unit time on the large islands should increase with island area; (3) the slope of the species–area

relationship should become steeper above the threshold. Because speciation is potentially a slow process, we also tested whether the number of *Anolis* species on large islands has reached speciation–extinction equilibrium.

Counts of reconstructed immigration and speciation events on a phylogeny underestimate the true number of events because lineages that have not survived to the present go unrecorded¹². However, if the rate of extinction of a lineage on an island does not depend on whether it immigrated or arose *in situ*, then the ratio of counts, when plotted against area, should correctly indicate the threshold island size above which local speciation exceeds immigration from outside as a source of new species. Figure 1 reveals that speciation is the dominant source of new species on islands greater than 3,000 km² (hereafter, ‘large’ islands). For example, the ten species on Puerto Rico (8,959 km²) are derived from three ancestral lineages that experienced seven *in situ* speciation events, and the seven species on Jamaica (11,425 km²) are derived from two colonizing species. Similarly, 42 of the 57 species on Cuba (114,524 km²) belong to two of the clades that have radiated on Cuba; at least 12 of the 15 other Cuban species are also the result of within-island speciation. In contrast, on small islands in the Caribbean, multi-species islands always harbour species that are distantly related and thus must have immigrated from elsewhere; of 143 islands smaller than 3,000 km² (hereafter, ‘small’ islands), no within-island speciation events were counted. On these smaller islands, speciation either does not occur, or its rate is too low to offset extinction. These results indicate an area threshold for speciation, in accord with the first prediction. Existence of such a threshold has been posited before in other taxa^{4,13}, but not previously quantified or documented using phylogenetic (sister species) criteria.

To test the second prediction we compared the number of recorded speciation events per unit time with island area using just the large islands. The number of recorded events by itself gives a biased estimate of speciation rate because some islands had more ancestral lineages than others (resulting from more immigration events or from more lineages present when islands fragmented) and

because it does not account for the different time periods that these lizards have been present on different islands^{9,14,15}. We used computer simulation to account for these two confounding factors. We simulated speciation using the phylogeny for Caribbean anoles in which branch lengths were scaled to relative time units. The result is clear: the recorded number of speciation events is positively related to island area (Fig. 2), suggesting that speciation rate itself scales positively with area.

Extinction rate decreases with island area in other taxa^{13,16–18} and might alone produce an apparent pattern of increased speciation rate on large islands even if the true rate of speciation is invariant. However, a declining extinction rate with increasing area is unlikely to be a sufficient explanation for the pattern in Fig. 2. First, estimates of speciation and extinction rates over all the large islands suggest that the effects of extinction are small (see below). Second, a strong relationship exists between island area and the number of species in ‘superspecies complexes’. Superspecies complexes are groups of closely related allopatric or parapatric species that differ little in morphology or ecology and are regarded as an early stage in allopatric speciation^{19,20}. If the rate of speciation were comparable among islands, we would expect to see a similar proportion of the species on each island belonging to superspecies complexes, but this is not the case. More than half (56%) of the species on Cuba are members of superspecies complexes, and Hispaniola’s proportion is nearly that high (45%). By contrast, no superspecies complexes are present on the two smaller islands. This result implies that at least one mode of speciation—allopatric speciation—is continuous and frequent on the largest islands, but rare on the smaller large islands. A third line of evidence supporting the presence of higher speciation rates on larger islands comes from changes in the slope of species–area relationships, discussed next.

The third prediction is that the slope of the evolutionary species–area relationship should be higher than that resulting when immigration is the sole source of new species. This prediction stems from the idea that speciation rate should increase with island area, in contrast to the weak expected effect of island area on immigration rate^{5,13,18}. An initially high rate of species accumulation on large islands is expected to lead to an even greater rate of speciation there because speciation is multiplicative and its rate per unit time rises

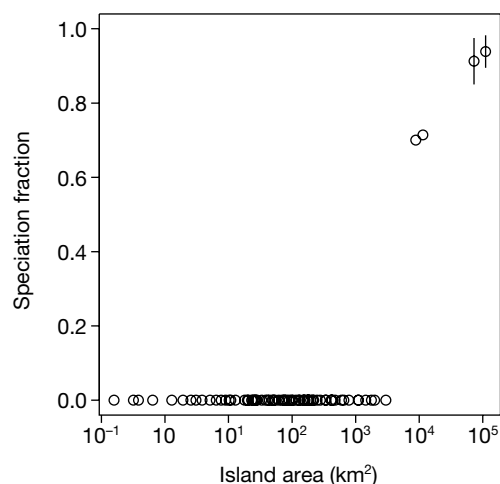


Figure 1 Recorded speciation events on islands, as a proportion of the total number of speciation and immigration events, in relation to island area. ‘Immigration’ probably includes some between-island speciation events resulting from island fragmentation (vicariance). Vertical bars represent the range of estimates resulting from different phylogenetic geographic reconstructions of the occurrence of ancestral taxa (see Methods). No ambiguity exists in the reconstructions for Puerto Rico and Jamaica; hence, each estimate is a single point. The increase in the speciation fraction with area is significant, according to a logistic regression ($\chi^2 = 26.8$, degrees of freedom, d.f. = 1, $P < 0.0001$; tested using the midpoints of vertical ranges).

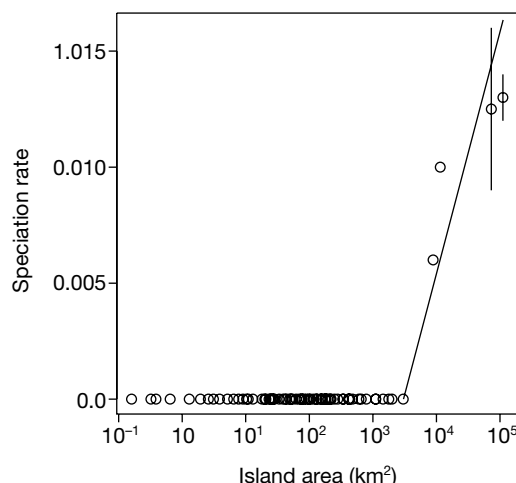


Figure 2 Speciation rate on large islands in relation to island area. Error bars as in Fig. 1. Regardless of which estimates are used, a relationship exists between island area (log-transformed) and speciation rate; the line shown here is based on an analysis using the midpoints of the vertical ranges ($F_{1,3} = 49.16$, $P = 0.006$), but any of the reconstructed values yields significant results. Analyses assumed a speciation threshold (x intercept) of 3,000 km² (compare Fig. 1) and no extinction. Branch lengths in the phylogeny were not calibrated against time, so there are no units for the y axis.

with the number of lineages present. The expected result is a steep species–area relationship when islands of different size are compared. Such a transition in slope is observed among the Caribbean islands (Fig. 3). A two-slope regression fits the species–area data far better than a single slope ($F_{2,143} = 34.12$, $P < 0.0001$), with the maximum likelihood breakpoint occurring near the island size at which speciation begins to exceed immigration as the source of new species (Fig. 1).

Several of the arguments above rest on the assumption that speciation exceeds extinction on the large Caribbean islands. We estimated overall rates of speciation and extinction by fitting the branching phylogeny for species on large islands to a constant birth–death process¹². Because our tree was incomplete, we used only the first 33 branching events (34 lineages), counted up from the root, which corresponded to approximately the first half of the total time span of the radiation. We assume that almost no branches are missing from this early period. This assumption is justified because the species selected to estimate the phylogeny were not sampled randomly, but rather were chosen to represent each of the major taxonomic series (clades) present in the archipelago. We therefore expect the vast majority of missing branches in the tree to occur after the first 33 branching events, towards the present time. To fit the birth–death process we also made use of the information provided by the present-day number of species descended from each of the initial 34 lineages. The best fit was a model in which species number increased exponentially through time and extinction was absent (Table 1). Confidence limits show that the data are also consistent with a non-zero (positive) extinction rate, but this rate does not approach that for speciation. The result suggests that

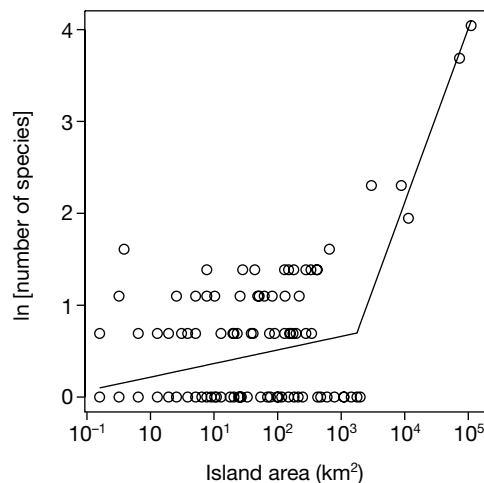


Figure 3 The species–area relationship fitted with a regression model having a breakpoint and two slopes. Slopes b_1 and b_2 were estimated using a nonlinear regression package that fitted the equation $\log(y) = a + b_1 \log(x) + b_2 [\log(x) - c] \delta$ to the data, where δ is an indicator variable equalling 1 when $x > c$ and 0 otherwise, a is the intercept and c is the breakpoint. Estimated slopes were $b_1 = 0.06 (\pm 0.02, \text{standard error of the mean, s.e.m.})$ and $b_2 = 0.76 (\pm 0.09, \text{s.e.m.})$.

Table 1 Estimated parameters of a birth and death process fit to the phylogeny of *Anolis* lizards on the four largest Caribbean islands

Parameters	Maximum likelihood estimate	Likelihood limits
Speciation – extinction	12.47	(9.44, 15.04)
Extinction/speciation	0.00	(0, 0.38)

Branches leading to other islands were pruned from the tree of ref. 9 before calculating. Two parameters were estimated using maximum likelihood¹². The first is the difference between the rates of speciation (birth) and extinction (death). The second is the ratio of extinction and speciation. The maximum likelihood estimates indicate that extinction is rare and speciation is the dominant process. Likelihood limits are parameter ranges whose log-likelihood is within 2 units of the maximum (equivalent to 95% confidence intervals).

after more than 30 million years of adaptive radiation by *Anolis* on Caribbean islands, species numbers are still increasing and fall below the speciation–extinction equilibrium.

Our results support the central tenet of the evolutionary theory of species–area relationships, that speciation rate increases with area. What mechanisms underlie this effect in *Anolis*? One likely explanation is that the opportunity for geographic isolation increases with island area. Hispaniola and Cuba are dissected by mountain ranges and many species are endemic to a single range. The largest islands have also been broken up into multiple smaller islands at times of higher sea level^{21,22}. It is also possible that the diversity of habitats increases with area²³, providing more opportunities for speciation by divergent natural selection²⁴. A relationship between area and habitat diversity is one aspect of ecological theories of species–area relationships, and it may likewise play a role in evolutionary theories of species diversity. However, an explanation based on a link between habitat diversity and speciation rate may be insufficient by itself here because some of the larger of the small islands (such as Guadeloupe, with one *Anolis* species, and Martinique, also with one *Anolis* species) are old and topographically and climatically diverse, yet have not experienced any speciation at all. Furthermore, among the Greater Antilles, Puerto Rico is a very old island with great vegetational and topographic diversity, yet it has experienced many fewer speciation events than Hispaniola and Cuba.

MacArthur and Wilson¹ offered Caribbean island reptiles as a primary example of the positive species–area relationship, but their ecological theory does not fully account for this relationship if within-island speciation is the source of many new species. We have shown that *Anolis* lizards of large islands require an evolutionary theory of diversity instead. This extension to ecological theories of island diversity is unlikely to be unique. For example, intra-island speciation is an important source of new species in many Hawaiian taxa including the drosophilids²⁵ as well as in reptiles on isolated Pacific islands²⁶. Lizard taxa on large islands elsewhere in the world (for example, Sri Lanka and Taiwan) have not experienced evolutionary radiations comparable to that of the Caribbean anoles, but their diversities might nevertheless follow similar patterns. Consequently, studies of speciation in relation to area would be well worth pursuing in other taxa and regions, as they would reveal the spatial context of speciation and the diversity of processes leading to the positive species–area relationship. □

Methods

Phylogeny

The phylogeny of *Anolis* is based on 1,455 base pairs of mitochondrial DNA for 53 species, 48 of which occur in the Caribbean (see Fig. 12a in ref. 9). To estimate the timing of divergence events on this phylogeny, we calculated branch lengths using maximum likelihood with the constraint that the total branch length from each species to the root of the phylogeny is equal, using DNAMLK in PHYLIP²⁷. To determine the validity of these branch lengths, we also calculated maximum likelihood branch lengths without the constraint that all species be equally distant from the root, using DNAML in PHYLIP. Values for branch lengths calculated in these two ways were highly correlated ($r = 0.94$), which indicates that the DNA data provides a reasonable estimate of the relative timing of divergence events. In addition, we examined residuals of the regression of constrained branch lengths on unconstrained branch lengths to determine whether there was any island bias resulting from the assumption of equal distance to the root (that is, did this constraint cause branches for taxa on some Greater Antillean islands to be shortened or lengthened relative to branches for taxa on other Greater Antillean islands?). No evidence for a bias was found (analysis of variance, $P > 0.84$), regardless of whether branches whose geographic assignment was equivocal, as discussed below, were assigned to either Cuba or Hispaniola.

Geographic inference of within-island speciation

Our data base^{10,11} included species occurrences on 147 Caribbean islands ranging in size from 0.15 to more than 100,000 km² (our database does not include species introduced by humans or islands that were connected to mainland America when sea levels were lower during the last ice age). To count the number of speciation events on an island, we assumed that the presence of sister taxa on an island resulted from speciation *in situ*. We consider the alternative that an island was colonized several times, followed by the extinction of ancestral species on other islands, to be far less likely. We also used information on

membership in species groups to assign speciation events to islands. Previous researchers assigned all Caribbean species to species groups (series); recent phylogenetic work² confirms that the species groups are almost invariably monophyletic. Therefore, if all species within a species group occur on a single island, then all speciation events within that species group were assumed to have occurred on that island, even those involving species not included in the phylogeny. In a few cases all members of a species group occur on a single island, with the exception of obviously recent dispersers to other islands (for example, all 15 species of the *sagrei* series occur on Cuba, with the exception of some populations of *A. sagrei* that occur on other islands); in these cases, we considered all species on the primary island to have arisen by within-island speciation. The phylogenetic affinities of two rare Hispaniolan species are unknown and these species were not included in our analyses.

On all landbridge islands near the Greater Antilles and the Bahamas, co-occurring species are always more closely related to species on other islands and thus do not provide evidence for within-island speciation. The one equivocal case involves two members of the *equestris* series that occur on Santa Maria, off the northern coast of Cuba. Whether these species are sister taxa remains to be determined. Again with one exception, all species on small nonlandbridge (oceanic) islands also belong to different species groups. The one exception is the two species on the Lesser Antillean island of St Vincent, whose sister taxa status is controversial²⁸. In addition, sympatric species on islands in the northern Lesser Antilles belong to the same species group (the *bimaculatus* series), but each island is occupied by one species from each of the two distinct subclades within the series²⁹. Because the *bimaculatus* series is monophyletic, it is conceivable that these two subclades initially arose by within-island speciation on one island, but we consider the alternative of allopatric differentiation on different islands to be more plausible. In summary, within-island speciation occurs very rarely or not at all on small islands. We do not consider any of the three possible exceptions as representing strong cases for within-island speciation. However, even if we had included them in our analyses, they would not have altered our conclusions.

Estimation of rate of speciation and extinction

We used computer simulation to test our prediction that number of recorded speciation events on large islands should correlate with area. To carry out the simulation, we used parsimony to infer inter-island immigration events and to determine the relative date on which new lineages immigrated to islands. In the most parsimonious reconstruction, Hispaniola was the ancestral locality for much of the anole radiation from which lineages on Cuba were derived independently several times. To examine the robustness of our analyses, we included the slightly less parsimonious (14 versus 15 steps) alternative possibility that Cuba was ancestral and that Hispaniola had been occupied independently by seven different lineages³⁰. Each simulation run began at the time, as indicated by the phylogeny, that the first species to a given island was recorded. In each time interval, all species present on an island had a probability, r , of speciating, thus increasing the number of species on the island by one. Additional species were added to the island at the times at which new lineages appeared (presumably by immigration or possibly by vicariance as island blocks collided^{21,22}), again as indicated by the phylogeny. In this way, the effect of the addition of new lineages to an island was incorporated into speciation rate estimates. Five hundred simulation runs were conducted and the mean number of species produced was calculated. Simulation trials were conducted iteratively, changing the value of r , until the mean number of species produced converged on the actual number of species that occurs on that island.

We used a modification of the likelihood method of ref. 12 to fit the *Anolis* phylogeny to a birth and death process. Our likelihood for birth and death parameters was the product of two parts. The first part is equation (17) of ref. 12 and is the probability density of the 32 observed waiting times between successive branching events of the phylogeny from the first branching event near the root to the 33rd branching event near the half-way point. The second part of the likelihood is based on equation (11) of ref. 12 and is the product of the probabilities that each lineage i living after the 33rd branching event has exactly k_i species at the present time, where k_i is its observed number of descendants, $k_i > 0$, and $i = 1, 2, \dots, 34$. For each lineage i this probability is $(1 - \eta_i)\eta_i^{k_i - 1}$ where t is the time between the 33rd branching event and the present time, $\eta_i = (\exp(rt) - 1)/(\exp(rt) - a)$, $r = (\text{speciation} - \text{extinction})$ and $a = \text{extinction/speciation}$.

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Fluorescent pigments in corals are photoprotective

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All reef-forming corals depend on the photosynthesis performed by their algal symbiont, and such corals are therefore restricted to the photic zone. The intensity of light in this zone declines over several orders of magnitude—from high and damaging levels at the surface to extreme shade conditions at the lower limit¹. The ability of corals to tolerate this range implies effective mechanisms for light acclimation and adaptation². Here we show that the fluorescent pigments^{3–9} (FPs) of corals provide a photobiological system for regulating the light environment of coral host tissue. Previous studies have suggested that under low light, FPs may enhance light availability^{4,5}. We now report that in excessive sunlight FPs are photoprotective; they achieve this by dissipating excess energy at wavelengths of low photosynthetic activity, as well as by reflecting of visible and infrared light by FP-containing

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chromatophores. We also show that FPs enhance the resistance to mass bleaching of corals during periods of heat stress, which has implications for the effect of environmental stress on the diversity of reef-building corals, such as enhanced survival of a broad range of corals allowing maintenance of habitat diversity.

The bright colours of corals (Scleractinia) and other Anthozoa are due to pigments of animal-host origin³, many of which are intensely fluorescent under ultraviolet-A (UVA) and blue light, with emission maxima at 420–620 nm (refs 4–9; Fig. 1). In many corals, distinct morphs are found that differ greatly in their concentration of FPs. Fluorescent proteins are part of a group of coral pigments for which the generic term ‘pocilloporins’ has been proposed^{9,10}; this group includes both brightly coloured, low-fluorescence forms and the highly fluorescent forms⁹ described here. Both types of pigments are partially homologous to green fluorescent protein (GFP)^{8,9}, first found in the luminescent jellyfish *Aequorea*¹¹ and widely used in cell biology. Although the function of GFP in luminescent systems is known, the role of similar FPs in non-luminescent anthozoans has hitherto been unclear^{6,10,12}.

We surveyed the distribution of fluorescent corals on the Great Barrier Reef (GBR; see Methods) and found that 124 species of 56 genera in 16 sampled families contained fluorescent morphs, often found growing side-by-side with non-fluorescent morphs. Colour polymorphism is typical of corals^{12,13}, but many FPs are invisible in daylight, so this widespread abundance has not been previously

reported. The highest numbers of fluorescent morphs were recorded at the shallowest sites; thus, 97% of sampled reef-flat corals at Heron Island, southern GBR, contained medium or high FP concentrations. Moreover, the relative concentration of FPs was significantly higher in parts of the colonies that were exposed to sunlight as compared to those parts that were shaded ($P < 0.001$, t -test). We therefore explored an early suggestion³ that fluorescent pigments in shallow water corals might function in photoprotection, by comparing fluorescent and non-fluorescent morphs.

We found that the emission maxima of FPs ranged from blue to green to red (Fig. 1), which is consistent with studies on the isolated FP proteins of corals⁹. The shorter wavelength FPs were more abundant. Microscopically, we identified two broad groups: FPs bound within fluorescent pigment granules (FPGs; 0.2–8 μm in size) as reported previously^{3,5,7}; and inter- or intracellular FPs that were not enclosed in granules (CFPs). Most corals contained multiple FPG and CFP types. Correspondences between emission and excitation maxima of FPs that occur in close association (Fig. 1e, f) suggest that energy transformation to longer, non-photosynthetically active, wavelengths might in some cases be a sequential process, with the fluorescence of one pigment exciting another, as expected from spectra of isolated proteins^{8,9}. We showed that this process could occur by comparing fluorescence of green FPGs (excitation maximum, 482.5 nm) alone and mixed with blue FPGs (excitation maximum, 382.5 nm). Only weak green fluorescence is seen under 330–380 nm excitation; addition of blue FPGs emitting at 480 nm enhanced green fluorescence intensity by 4–17 times. (The effect was strongly dependent on distance, with maximal enhancement when blue and green granules were less than 10 μm apart.) The final energy spill (the wavelength of the energy at the end of the pigment coupling process) would then, depending on the pigments involved, lie between the two main peaks of the coral photosynthetic action spectrum and hence be relatively inactive in photosynthesis (Fig. 1f). This would be the inverse counterpart of the process of light transfer to photosynthesis that others have proposed for light-limited habitats^{4,5} (Fig. 1g).

High levels of light cause photodamage and photoinhibition¹⁴ in coral symbionts^{15–19}. We considered that FPs may reduce the susceptibility to photoinhibition of fluorescent corals by filtering out damaging UVA and excessive photosynthetically active radiation (PAR). We compared the degree of daytime photoinhibition in a polymorphic intertidal species, *Acropora palifera*, by exposing replicate sub-colonies made from green fluorescent, brown medium fluorescent and beige non-fluorescent mother colonies to full sunlight, and monitored photosynthesis by chlorophyll fluorescence

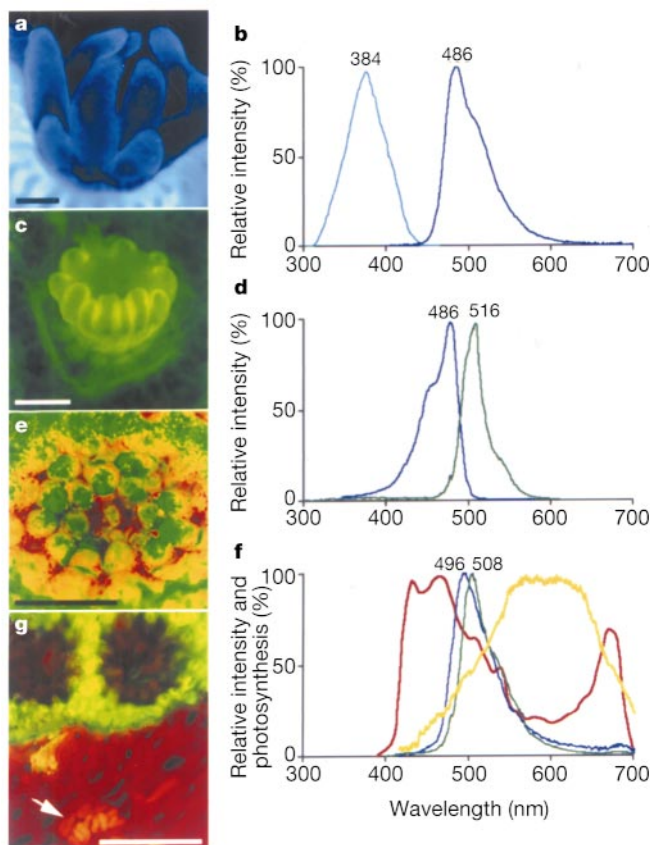


Figure 1 Main types of fluorescent pigments (FPs) in coral polyps. These pigments are found in blue, green, yellow and red combinations (a, c, e, g) with overlapping excitation and emission spectra (b, d, f). a, b, Mainly blue, in *Acropora nobilis*. c, d, Mainly green, in *Pocillopora damicornis*. e, f, Emissions of outer blue/green and underlying yellow FPs in ‘sun’ *Porites cylindrica*. Coral photosynthetic action spectrum²⁴ (red line) shows that much of the energy is emitted at wavelengths not usable in photosynthesis. g, Sub-surface red FPs in green *Montipora digitata*. Arrow, red FPs in mesenteric filaments. Scale bars, 0.5 mm. ‘Sun’ refers to corals from high-sunlight habitats, ‘shade’ refers to corals or parts of corals from shaded habitats.

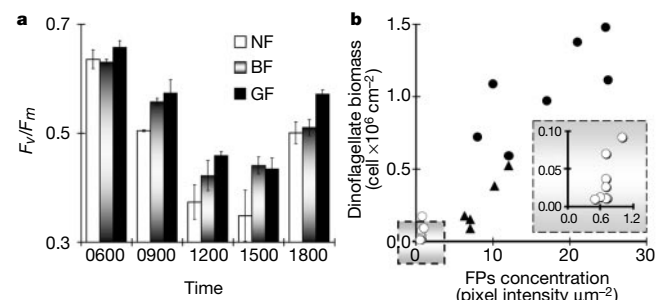


Figure 2 Photoinhibition and bleaching responses of corals. a, Maximal potential quantum yield (F_v/F_m) of dinoflagellates in green highly fluorescent (GF), brown medium fluorescent (BF) and non-fluorescent (NF) *Acropora palifera*. Results are means $\pm$ s.e. for 3 sub-colonies $\times$ morph $\times$ 2 tanks $\times$ time interval (that is, the samples (270) taken from 3 colonies for each of the 3 morphs, divided randomly between 2 tanks, and killed for PAM fluorescence measurement at 5 given times). b, Dinoflagellates per cm^2 and relative concentration of fluorescent pigments per μm^2 of sampled corals: open circles, bleached; triangles, part-bleached; filled circles, unbleached. Inset, enlarged section of graph marked in square.

analysis with a pulse amplitude modulation (PAM) fluorometer^{19,20}. As expected^{15,16}, corals showed pronounced photoinhibition during periods of peak irradiance; non-fluorescent morphs, however, were significantly more photoinhibited and recovered to pre-inhibition rates slower than fluorescent morphs ($P < 0.001$, analysis of variance) (Fig. 2a). Similar measurements with other polymorphic species (*Acropora nobilis*, *Pocillopora damicornis*, *Goniastrea retiformis*) also indicated that FPs are correlated with reduced photoinhibition.

As high solar radiation is a factor in the widely observed mass bleaching of corals^{17,21,22}, FPs might affect susceptibility to bleaching. Bleaching occurs as a consequence of damage to dinoflagellate photosynthesis caused by the combined effects of thermal stress and sunlight^{18,19,22}; the dinoflagellates either degrade or are expelled from the host. During the severe 1998 mass bleaching event on the GBR, we sampled 21 common coral species affected by bleaching to varying degrees. We found a significant correlation ($r^2 = 0.9471$; $P < 0.0001$) between bleaching resistance (that is, high tissue dinoflagellate biomass) and the concentration of FPs within the tissue (Fig. 2b).

Light scattering is an important factor linked to the sun-screening function of FPs. We measured spectral reflective properties of coral tissues with fibre-optic microprobes^{23,24} positioned over specific parts of single coral polyps. The highly reflective bare coral skeleton was used as a reflection standard (100%). FPs greatly modified the surface light environment, not only by their emissions but also by light scattering and reflectance, which was higher in areas with high FPG concentrations (Fig. 3a). White-pigmented regions of tissues,

formed by dense layers of FP chromatophores, had 60–100% reflectivity (Fig. 3a, b). The most pigmented, and most reflective, parts of colonies—first, branch tips and colony edges, and second, the oral disk/cone and tentacle tips, which on polyp retraction form a sun-screening polyp ‘plug’⁷ (Fig. 4c)—correspond respectively to known areas of highest cell division and areas immediately above reproductive organs. This distribution points to a photoprotective role of FPs in screening sensitive coral tissues as well as symbionts.

Our observations also indicate that corals actively vary the areal density of pigment chromatophores by polyp expansion and contraction. During expansion, more light penetrates into the tissues through the gaps between FPGs. Under high light, polyp contraction leads to denser concentration of tissue FPGs and cytoplasmic FPs, forming a thicker and a quasi-continuous FP layer; this layer acts as an effective sunscreen (Fig. 3b, c) by light scattering and by radiant fluorescence energy transfer from shorter to longer wavelengths. We also found that FP-containing polyps of shade-adapted and high-light-adapted corals exhibited differences in spectral reflectivity. Shade-adapted polyps absorbed most of the incident light, in line with previous observations²⁵, whereas polyps that had adapted to high light levels were generally 20–100% more reflective (Fig. 3b).

What are the causes of such different optical properties of tissue from shade- and high-light-adapted corals? The three-dimensional (3D) cellular localization of FPs in corals showed a clear difference in the distribution of FPs relative to the layers of endosymbionts⁷. In high-light-acclimated corals, FPs are localized above the endosymbionts (Fig. 4a–c), and are in a position to screen them from excess sunlight. In shade-adapted corals from light-limited habitats, FPs are localized endodermally, among or below the layers of endosymbionts (Fig. 4d), consistent with their proposed function of light enhancement for photosynthesis by way of wavelength-transformation and back-scattering^{4,5}.

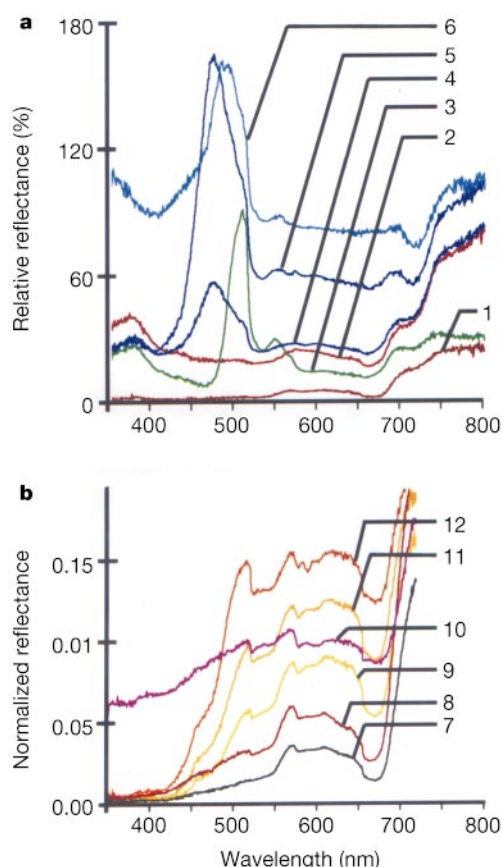


Figure 3 Apparent reflectance. **a**, *Plesiastrea versipora*: tissues lacking FPs (1); tissue with blue FPs overlying skeletal ridge (2); contracted tentacle with green FPGs (3); blue FPGs in expanded (4) and contracted (5) oral disk; dense blue FPGs in white oral disk of shallow-water *Platygyra daedalea* (6). **b**, Intertidal yellow *Porites cylindrica*: ‘shade’ expanded (7); contracted (8); and ‘sun’ expanded (11), contracted (12) tentacles; edge of polyp calyx (9); septal skeleton with thin tissue (10). Dips in spectra are due to absorption by photosynthetic pigments. Spectral peaks are due to FPs emission and reflection.

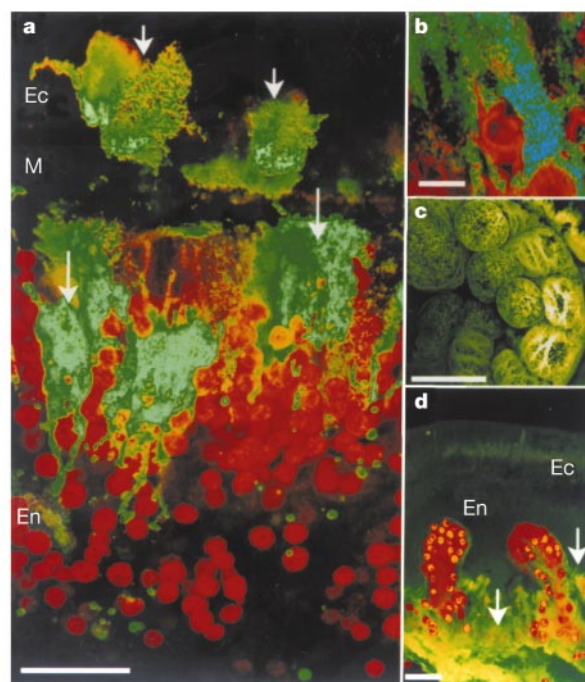


Figure 4 Reconstruction of serial confocal sections through tissues of ‘sun’ and ‘shade’ corals. **a**, FPs in chromatophores above endosymbionts in polyp tentacle of ‘sun’ intertidal *Goniopora tenuidens*. Scale bar, 50 μm . **b**, FPG-filled chromatophores with long extensions enveloping endosymbionts. Scale bar, 10 μm . **c**, Retracted tentacles with dense FPGs form a ‘plug’ over polyp. Scale bar, 1 mm. **d**, Endodermal FPs below dinoflagellates in ‘shade’ *Lobophyllia corymbosa*. Scale bar, 50 μm . FPs shown in green/yellow, symbiotic dinoflagellates in red. Arrows, FP chromatophores. Ec, ectoderm (epidermis); M, mesoglea; En, endoderm (gastrodermis).

The present results suggest that FPs reduce the photoinhibitory effect of high levels of solar radiation, which in conjunction with thermal stress leads to bleaching. These findings improve our understanding of the causes of observed inter- and intraspecific variability in bleaching^{17,26}, and may provide an insight into how changing global climate conditions would influence the species diversity and rate of change of coral reef communities²².

The evidence presented here indicates that the role of FPs in photoprotection in shallow water, hitherto neglected, is at least as significant as the function of light capture in deep water previously assigned to them. Dinoflagellate photosynthesis is vulnerable to both UV^{27,28} and high levels of PAR^{16–19}. While accessory pigments in dinoflagellates can dissipate excess PAR as heat²⁹, FPs can dissipate excess light energy through fluorescence and light scattering. FPs may also supplement screening of UV radiation by mycosporin-like amino acids³⁰, as some FPs can transform absorbed UVA radiation to longer non-actinic wavelengths through fluorescence. By screening chlorophylls and peridinin from high levels of solar radiation and by absorbing UVA, FPs thereby decrease the likelihood of irreversible photoinhibition, photooxidation and subsequent coral bleaching. By changing their optical properties with the help of these GFP-like pigments, coral polyps are able to optimize the photosynthetic activity of their tissues for the better survival of the organism. □

Methods

Survey sites, sampling and manipulations

Surveys of fluorescent corals were made at the inter-tidal lagoon, reef flat and inner and outer slope of Heron Island (23° 26' S, 151° 55' E) and One Tree Island (OTI) (23° 30' S, 152° 06' E), and at several GBR mid-shelf-reefs (1–20 m depth). Corals were sampled by chiselling pieces from replicate colonies ($n = 3–6$). Each sample was broken in two, and subsequently, one subsample was frozen and the other was chemically fixed as described previously⁷ for microscopy. Polymorphic *A. palifera* colonies used in photoinhibition experiments were collected from the OTI lagoon from ~1 m depth. Three colonies of each colour morph were broken into replicate sub-colonies, and fixed in horizontal position in flowing sea water (27–28 °C) with one side exposed to sunlight and the other shaded. Controls were kept at 50–80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. During the March 1998 bleaching event, samples were taken at Coats (17° 28' S, 146° 30' E) and Cayley (18° 30' S, 147° E) mid-shelf reefs from depths of 1–6 m. Replicate samples ($n = 3–6$) were taken from species selected by susceptibility to bleaching: 9 bleached (including non-fluorescent *A. nobilis* morph); 5 partially bleached (including fluorescent *A. nobilis*); 7 unbleached.

Microscopy

Frozen, glutaraldehyde-fixed and live coral samples were analysed by fluorescence wide-field and confocal (CLSM) microscopy as described previously⁷. Fluorescence characteristics of FPs were not substantially affected by glutaraldehyde fixation. Confocal imaging used 488-nm excitation, with detection at 520–550 nm (FPs) and >585 nm (chlorophyll). 3D reconstruction from optical sections was done with VoxelView Ultra 2.1.2 (Vital Images). A fluorescence microscope fitted with a cooled CCD camera (PCO Sensicam) was used to test energy transfer from blue to green FPGs, both extracted from *Plesiastrea versipora*. Intensity (in the green) was measured as the ratio of emission excited at 330–380 nm to the emission excited at 450–490 nm, thereby compensating for differences between granules. Relative FP concentrations in light- and shade-adapted samples—as well as in post-bleaching samples—were measured semi-quantitatively by CLSM as the fluorescence intensity per μm^2 of imaged coral surface (replicate 3–6 colonies per specimen). Zooxanthellae were extracted from samples, and their biomass per cm^2 of coral surface was determined microscopically and correlated to the relative concentration of FPs in surface tissue as determined by CLSM. Coral surface areas were measured as described previously¹⁸.

Spectroscopy

Fluorescence excitation and emission spectra of FPGs isolated by homogenization and repeated centrifugation of coral tissues in phosphate buffer (0.1 M, pH 7.2) were determined by a Perkin Elmer Luminescence Spectrometer S50B. All spectra were normalized to their peaks. Reflectance spectra were measured on live corals in sea water by a tapered (40- μm tip) fibre-optic field radiance microprobe²³ positioned ~100 μm above the coral surface. The microprobe was connected to a fibre-optic diode-array spectrometer (Hamamatsu PMA-11) with a 300–800 nm spectral range. A micromanipulator was used to position the microprobe tip above specific single polyp regions, as viewed under a dissection microscope. Samples were illuminated by a UV-visible metal halide light source through a 1-mm quartz fibre equipped with a collimator at the output end. Spectra of reflected light from the corals were normalized to the spectrum of reflected light from a reflectance standard in order to obtain reflectance spectra corrected for the spectral composition of incident light (normalized reflectance). Data were subsequently expressed as percentages of surface downwelling radiance reflected from a cleaned coral skeleton (relative reflectance).

Active fluorescence measurements

Throughout the day (06:00, 09:00, 12:00, 14:00, 18:00), photoinhibition of light-exposed and shaded portions of sub-colonies ($n = 3$ per morph), and shaded controls, was measured as decrease in the maximal potential quantum yield (F_v/F_m) of PSII by a pulse amplitude modulation fluorometer (DIVING-PAM)^{19,20} after 30 min dark-adaptation (where F_v is the variable fluorescence yield and F_m is the maximum dark-adapted fluorescence yield). Photosynthetically active radiation (400–700 nm) during the experiment was measured by an LI-190SA quantum sensor (Li-Cor Inc.).

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Conservation and elaboration of *Hox* gene regulation during evolution of the vertebrate head

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The comparison of *Hox* genes between vertebrates and their closest invertebrate relatives (amphioxus and ascidia) highlights two derived features of *Hox* genes in vertebrates: duplication of the *Hox* gene cluster^{1,2}, and an elaboration of *Hox* expression patterns and roles compared with non-vertebrate chordates^{3–8}. We

have investigated how new expression domains and their associated developmental functions evolved, by testing the *cis*-regulatory activity of genomic DNA fragments from the cephalochordate amphioxus *Hox* cluster in transgenic mouse and chick embryos. Here we present evidence for the conservation of *cis*-regulatory mechanisms controlling gene expression in the neural tube for half a billion years of evolution, including a dependence on retinoic acid signalling. We also identify amphioxus *Hox* gene regulatory elements that drive spatially localized expression in vertebrate neural crest cells, in derivatives of neurogenic placodes and in branchial arches, despite the fact that cephalochordates lack both neural crest and neurogenic placodes. This implies an elaboration of *cis*-regulatory elements in the *Hox* gene cluster of vertebrate ancestors during the evolution of craniofacial patterning.

New gene-expression sites and regulatory differences may have been important in permitting the evolution of increased body-plan complexity in vertebrates. The evolution of vertebrates involved new *Hox* expression in neural crest and mesoderm, in addition to refinement of expression domains to pattern the axial skeleton and nervous system. To investigate how vertebrate *Hox* gene regulation evolved, we examined the regulatory potential of genomic DNA from the amphioxus *Hox* cluster (*AmphiHox-1* to *AmphiHox-3*) in vertebrate embryos. These 3' genes are homologous to Hox paralog groups 1–3 of vertebrates, for which many of the modular elements involved in tissue-specific and spatially restricted expression are known^{9,10}. We linked amphioxus DNA fragments to a *lacZ* reporter (Fig. 1a, b) and assayed their regulatory activity in transgenic mice and in chick embryos; the latter used a method developed for focal *in ovo* electroporation¹¹. We found that four DNA fragments mediate consistent patterns of reporter expression in mouse and chick embryos.

Two regions, 1A and 3B, stimulated expression in the central nervous system (CNS) of vertebrate embryos (Fig. 2). Element 3B, 5' of *AmphiHox-3* (Fig. 1b), mediates expression with an anterior limit between rhombomere (r) 5 and r6 in mouse and chick embryos (Fig. 2e–g). Lower levels are detected in r6 than in r7, r8 and the spinal cord. This shows that *cis*-regulatory elements from

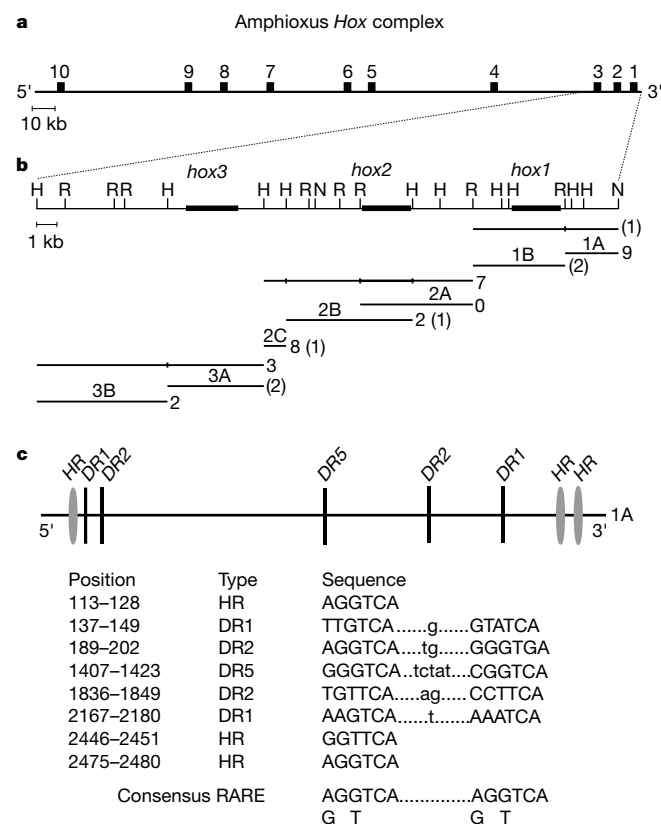


Figure 1 Transgenic analysis of the 3' end of the amphioxus *Hox* complex. **a**, The *AmphiHox* cluster. **b**, Genomic fragments used for transgenesis derived from the *AmphiHox* cluster. The names of genes (above) and the number of embryos showing a consistent expression pattern in mouse embryos (right) are shown. Numbers in parentheses indicate mouse embryos with ectopic, integration-dependant or non-reproducible patterns. **c**, Sequence analysis of element 1A. The position, type and sequence of multiple consensus RAREs is shown. DR_n, RARE direct repeat with *n* nucleotides of spacing; HR, half repeat; H, *Hind*III; N, *Not*I; R, *Eco*RI. The DNA sequences of elements 1A, 2B and 2C are deposited in the GenBank/EMBL/DBJ databases (accession numbers AB 050886–8).

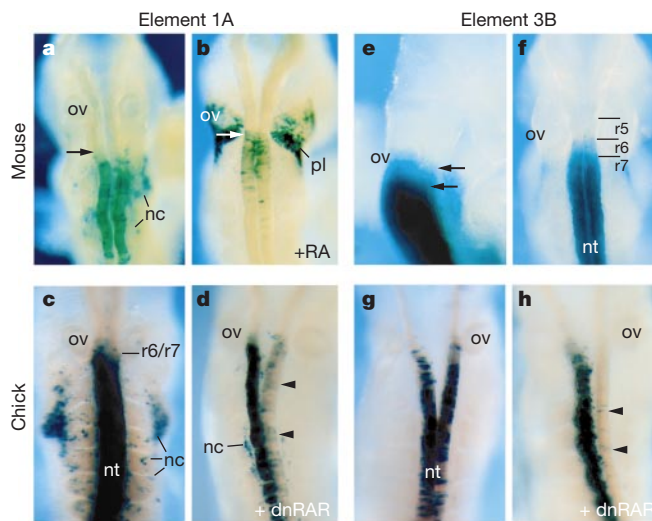


Figure 2 Neural expression mediated by amphioxus *Hox* regulatory elements in mouse and chick embryos. **a–d**, Element 1A drives expression in neural crest (nc) and up to the caudal hindbrain in mouse (**a, b**) and chick (**c, d**). **b**, RA treatment shifts expression anterior to r5/r6 (arrows) and induces otic placode (pl) expression. **d**, dnRAR down-regulates 1A reporter expression (right side). **e–h**, Element 3B drives expression to the r5/r6 boundary in mouse (**e, f**) and chick (**g, h**) embryos. **h**, The dnRAR abolishes 3B reporter expression in the chick neural tube (nt) (right side). Embryo stages: mouse, 9.5 d.p.c.; chick, HH14. ov, otic vesicle.

amphioxus *Hox* genes can be recognized by vertebrate embryonic cells and that tissue specificity is conserved, as neural tube is the only tissue in which *AmphiHox-3* is spatially expressed⁷. However, vertebrate group-3 genes are normally expressed one rhombomere anterior to the *AmphiHox3* reporter^{10,12}. This r5 expression is controlled by a *kreisler*-dependent segmental enhancer^{13,14}. We detected no such activity in amphioxus fragment 3B. Element 1A, 3' of *AmphiHox-1* (Fig. 1b), also mediates spatially restricted reporter expression in the neural tube of both mouse and chick embryos (Fig. 2a and c). The patterns are similar to those found for the endogenous *Hoxa-1* and *Hoxb-1* genes in both of these species^{12,15,16}, except that there is no r4-restricted domain. The r4 segmental pattern is controlled by a group 1 *Hox/Pbx*-dependent autoregulatory loop^{9,10,17}, and the amphioxus element 1A lacks these consensus binding sites. These results suggest that regulation of basal neural expression is conserved between amphioxus and vertebrates, but that elaboration of segment-specific patterns in vertebrates involved the addition of new elements.

Because retinoic acid (RA) is important for patterning and basal *Hox* gene regulation in the vertebrate neural tube^{17–19}, we investigated whether the amphioxus 1A and 3B neural elements are dependent on RA signalling. Sequence analysis of element 1A

shows the presence of many putative retinoic acid response elements (RAREs) (Fig. 1c), analogous to the multiple RAREs found at the 3' end of the vertebrate group 1 genes^{9,19}. We found that element 1A responds to exogenous RA, inducing an anterior shift in CNS expression and an upregulation in the otic placode (Fig. 2b). Conversely, by blocking normal RA signalling in chick embryos with a dominant-negative form of the retinoic acid receptor- α (dnRAR)^{11,18,20}, reporter staining was specifically downregulated on the transfected side (Fig. 2c, d). This shows that *in vivo* activity of element 1A is dependent on RA action and suggests that the RA response of *AmphiHox-1* in amphioxus embryos²¹ is mediated through element 1A. In addition, we investigated element 3B, as there is an RA-dependent enhancer in mouse located between *Hoxb-3* and *Hoxb-4* that acts on both genes and directs reporter expression in a similar pattern to that mediated by amphioxus element 3B (refs 18, 22; Fig. 2e, f). Element 3B is also dependent on RA signalling in the chick neural tube, as reporter activity is downregulated by dnRAR (Fig. 2g, h). Thus, the RA-dependent activity and relative position of neural enhancers from *Hox* paralogy

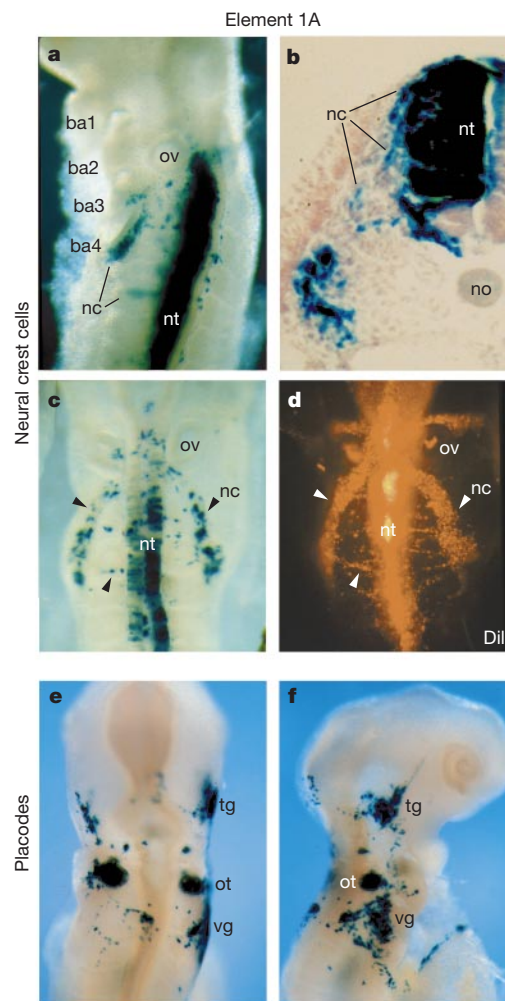


Figure 3 Element 1A directs expression in neural crest cells and placodes of chick embryos. **a**, Reporter expression in streams of neural crest (nc) cells migrating from the neural tube (nt) into branchial arches (ba) 3 and 4. **b**, Section showing nc cells migrating from the dorsal nt. **c**, **d**, Electroporation and Dil lineage tracing analysis. Dil-labelled nc cells migrate out of the nt (**d**, darkfield) and co-express the LacZ reporter (**c**, bright field). **e**, **f**, Electroporation into surface ectoderm, dorsal (**e**) and lateral (**f**) views. Staining is in trigeminal ganglion (tg), otic placode (ot) and vagal ganglion (vg). no, notochord; ov, otic vesicle.

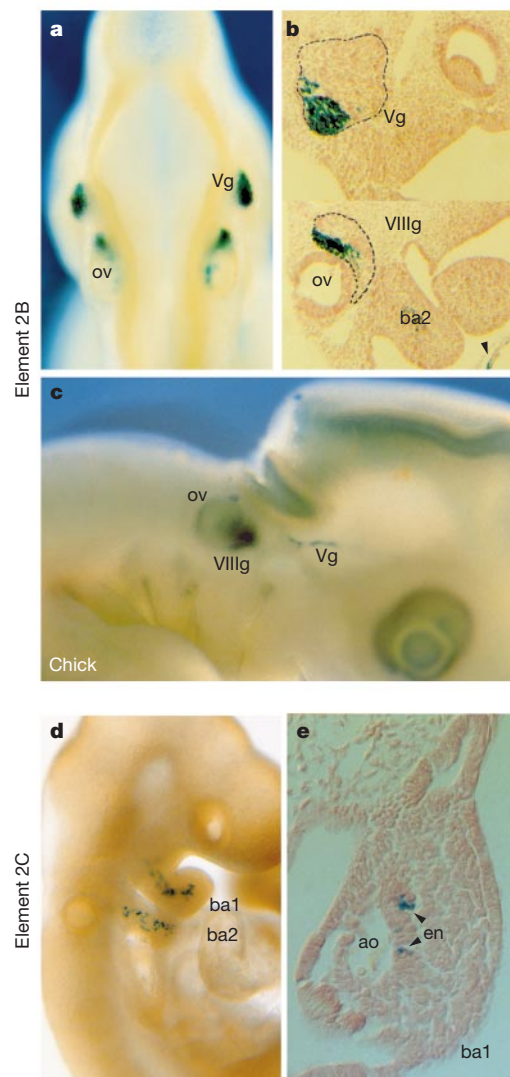


Figure 4 Expression in neurogenic placodal and mesodermal derivatives mediated by amphioxus *Hox* elements. **a–c**, Element 2B drives reporter expression in the trigeminal (Vg) and acoustic (VIIIg) ganglia in mouse (**a**, **b**) and chick (**c**) embryos. Expression is limited to the posterior regions of the ganglia (**b**). **d**, **e**, Branchial arch (ba) expression of Element 2C in mouse embryos. Staining is apparent in both ba1 and ba2 (**d**). Section through ba1 (**e**) shows expression in a subpopulation of mesodermally derived aortic endothelial cells (en). ao, aorta; ov, otic vesicle.

groups 1 and 3/4 are conserved between amphioxus and vertebrates.

Amphioxus lacks neural crest and neurogenic placodes, which are sites of endogenous *Hox* gene expression in vertebrates^{12,23}. Even so, amphioxus element 1A directs reporter expression in these tissues in both chick and mouse embryos (Figs 2a–d and 3). In the chick embryo, the reporter construct was introduced only into the neural tube, yet staining was detected in many cells streaming dorsally and laterally from the neural tube (Figs 2c, 3a–c). Using the lipophilic dye DiI as a lineage tracer, we labelled the dorsal region of the chick neural tube and detected co-expression of the reporter gene and DiI in lateral cell populations (Fig. 3c, d). This confirms that expression occurs in migratory neural crest cells derived from the neural tube. This contrasts with element 3B, which is strongly expressed in the neural tube but not in neural crest (Fig. 2). Furthermore, when element 1A was electroporated into the surface ectoderm, reporter expression was specifically localized in placode derivatives, such as the trigeminal and acoustic ganglion and the otic vesicles (Fig. 3e, f). This placodal expression is not blocked by the dnRAR construct (data not shown), which shows that unlike the neural tube and neural crest it is not dependent on RA signalling. Specific placodal enhancers have not yet been identified in vertebrate *Hox* clusters. Hence, DNA sequences 3' of *AmphiHox-1* contain elements that are capable of directing spatially restricted expression in vertebrate embryos in the neural tube, neural crest cells and placode derivatives.

Two fragments surrounding the *AmphiHox-2* locus show no activity equivalent to the conserved r3/r5 *Krox20*-dependent enhancers of *Hoxa-2* and *Hoxb-2* (refs 24–26). Fragment 2C (Fig. 1b) mediates a temporally dynamic pattern of expression in subsets of cells in the first and second branchial arches in transgenic mouse embryos (Fig. 4d, e). The location of cells in the aortic endothelium (Fig. 4e), and the lack of reporter expression in neural crest cells after electroporation of fragment 2C into the chick neural tube suggest that expression is occurring in mesodermal cells. In contrast, fragment 2B (Fig. 1b) mediates strong expression in mouse embryos in part of the trigeminal and facio-acoustic ganglia (Fig. 4a, b), which have a dual embryonic origin from neural crest and placode²⁷. To distinguish between these origins, we electroporated the construct separately into the surface ectoderm or the neural plate of chick embryos. Only gene transfer into the surface ectoderm results in detectable reporter expression in the same sensory ganglia (trigeminal and acoustic) as in the mouse (Fig. 4c); hence, the amphioxus 2B element activates restricted expression in derivatives of the dorsolateral neurogenic placodes independent of the neural crest and neural tube.

These results yield insights into the evolution of *Hox* gene regulation. First, the amphioxus *Hox* cluster contains a similar organization of RA-dependent neural enhancer elements that mediate basal aspects of *Hox* gene regulation conserved between amphioxus and vertebrates. This highlights the importance of retinoid signalling for *Hox* gene activation in chordates. Second, there are important differences in *Hox* regulation between amphioxus and vertebrates in the neural tube. We did not detect—by activity or by sequence—amphioxus counterparts of the vertebrate enhancers that impart rhombomere-specific *Hox* expression through the *kreisler* or *Krox20* elements, or the *Hox/Pbx*-dependent autoregulatory elements^{13,14,24–26}. We conclude that upregulation of *Hox* gene expression in specific rhombomeres probably reflects the addition of enhancer elements in the vertebrate lineage, in association with the emergence of hindbrain segmentation.

Our finding that *cis*-regulatory elements from amphioxus *Hox* genes mediate reporter expression in vertebrate neural crest cells (element 1A) and derivatives of neurogenic placodes (elements 1A and 2B) has important evolutionary implications. Migratory neural crest cells have not been reported from amphioxus, and are considered to be a vertebrate characteristic; indeed, derivatives of neural crest are defining features of vertebrates²⁸. Similarly, there is no evidence for homologues of neurogenic placodes in amphioxus.

One might have predicted, therefore, that new enhancer elements controlling *Hox* gene expression in neural crest cells and neurogenic placodes arose during vertebrate evolution. Instead, our results indicate that some *Hox* gene enhancers that are capable of directing expression in these tissues were already present in the common ancestor of amphioxus and vertebrates. Some of these regulatory regions, such as element 1A, may have directed expression in evolutionary precursor populations of neural crest and placodes and are thus revealing conserved roles. In contrast, results from element 2B, which mediates expression in placodal tissue independently of the neural tube and neural crest, indicate that some *Hox* gene enhancers may have been co-opted from existing regulatory elements with distinct roles in the ancestral chordate *Hox* cluster during the evolution of craniofacial patterning. Our work opens up new ways of examining the problem of how the *Hox* regulatory machinery is associated with the generation of neural crest and placode lineages during the appearance of the “new head”²⁸. □

Methods

Transgenic mice

Genomic DNA fragments derived from λ -clones that cover the end 3' 30 kilobases of the *AmphiHox* cluster were cloned in the BGZ40 reporter vector, which contains a minimal TATA human β -globin promoter, bacterial *lacZ* and an SV40 polyadenylation signal. We performed generation, reporter analysis and histology of transgenic embryos as described^{13,14,24–26}. We administered RA by oral gavage at 7.5 days post coitum (d.p.c.), and we assayed the inductive response at 9.5 d.p.c. as described¹⁸. We injected constructs initially as pools (1A + 1B), (2A + 2B + 2C) and (3A + 3B), and then later as individual constructs to screen for regulatory activity.

Chick electroporations

We injected DNA constructs designed for transgenic analysis into the closed chick neural tubes at the 8–10-somite stage, and then applied generally electric pulse to both sides as described¹¹. To target surface ectoderm, we placed the DNA solution on top of the embryo and the electrodes above and below. In cases where we performed co-electroporation with dnRAR vectors, we performed a second injection after the initial injection and electroporation to remove the original sample and replace it with a solution containing both of the DNAs. For some embryos, we injected DiI (0.5 mg ml⁻¹) after the electroporation to trace neural crest cell lineages. We cultured embryos for 24 h after the electroporation and processed them for further DiI or *lacZ* analysis.

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Performance monitoring by the supplementary eye field

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Intelligent behaviour requires self-control based on the consequences of actions. The countermanding task is designed to study self-control; it requires subjects to withhold planned movements in response to an imperative stop signal, which they can do with varying success. In humans, the medial frontal cortex has been implicated in the supervisory control of action^{1–3}. In monkeys, the supplementary eye field in the dorsomedial frontal cortex is involved in producing eye movements, but its precise function has not been clarified⁴. To investigate the role of the supplementary eye field in the control of eye movements, we recorded neural activity in macaque monkeys trained to perform an eye movement countermanding task. Distinct groups of neurons were active after errors, after successful withholding of a partially prepared movement, or in association with reinforcement. These three forms of activation could not be explained by sensory or motor factors. Our results lead us to put forward the hypothesis that the supplementary eye field contributes to monitoring the context and consequences of eye movements.

To evaluate the hypothesis that neurons in the supplementary eye field (SEF) register the consequences of actions, we collected behavioural and neurophysiological data from two macaque monkeys performing an eye movement countermanding task that manipulated their ability to withhold planned movements^{5,6}. On trials with no stop signal, the monkeys received positive reinforcement

following a saccade to the target. On trials with a stop signal, the monkeys earned reinforcement when the partially prepared saccade to the target was cancelled and fixation was maintained. The task is described in detail in Fig. 1 of Supplementary Information. The delay of the stop signal relative to the target was adjusted such that monkeys failed to cancel the saccade in half of the trials. We analysed the distribution of reaction times and the probability of cancelling the movement to determine the time needed to cancel the planned movement—the stop-signal reaction time^{5,6}.

In the two monkeys, 175 neurons (monkey A, 58; H, 117) recorded in the SEF provided sufficient data during the countermanding task for us to analyse. Figure 2 of Supplementary Information shows the location of the recordings. Neurons were classified by the presence of visual, movement and postsaccadic activity. The hypothesis was tested by comparing the activity between stop-signal trials with cancelled or non-cancelled movements and the activity observed in trials with no stop signal. Here we focus on three types of neurons. Neurons of the first type (26/175; 15%) were modulated specifically when monkeys failed to cancel the planned movement (Fig. 1). Neurons of the second type (23/175; 13%) were modulated specifically when monkeys successfully cancelled planned movements (Fig. 2). Neurons of the third type (39/175; 22%) were active before and during the delivery of reinforcement (see below).

Could this neural activation in the SEF be explained by sensory or motor factors? The modulation during countermanding trials was compared to the presence of visual, movement or postsaccadic activity during memory-guided saccades, but none of the three types of neurons could be uniquely identified with a previously described cell type in the SEF. Table 1 in Supplementary Information gives the incidence of visual and saccade-related activity for these types of neurons.

Several observations indicate that the first type of neural modulation is not exclusively of sensory origin even though different patterns of visual stimulation occurred in non-cancelled trials and in trials without the stop signal (Fig. 1). First, if the modulation were a visual off-response, then these neurons should respond to the disappearance of the fixation spot in trials with no stop signal or to the disappearance of the target in memory-guided saccade trials, but they did not. Second, if the modulation were a visual on-response to the stop signal, it should occur during successfully cancelled trials, but it did not. Finally, in additional testing of a subset of neurons of this type ($n = 12$), the modulation was observed even if the visual stimulation after the non-cancelled saccade was identical to that in trials with no stop signal. Other observations indicate that modulation of the first type was not responsible for producing movements and is distinct from the postsaccadic activity in the frontal eye field (FEF). First, the modulation was not observed after movements in trials without a stop signal even though the metrics and dynamics of non-cancelled saccades are not different from saccades in trials without a stop signal⁶. Second, the modulation occurred after both contraversive and ipsiversive saccades whereas SEF neurons have mainly contralateral movement fields⁷. Third, the modulation had no relation to the gaze behaviour after the error, occurring with equal magnitude whether monkeys continued fixating the target or shifted gaze to the location of the fixation spot or elsewhere. The inability to explain this modulation in terms of retinal stimulation or eye movements allows the possibility that it signals the occurrence of an error. Evidence supporting this interpretation is the observation that the putative error-related activity in the SEF occupied the same interval as error-related potentials originating in medial frontal cortex^{8–13} (Fig. 1c).

Several observations lead to a different interpretation of the second type of modulation (Fig. 2). It cannot be a visual response to the stop signal because it did not happen in non-cancelled trials. The activation could not contribute to cancelling the movement because it occurred after the stop-signal reaction time (Fig. 3a). To determine how this modulation related to performance, its magnitude was

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quantified as a function of both the stop-signal delay and the probability of not cancelling the movement. The strength of this modulation did not vary with stop-signal delay. However, for a given stop-signal delay the probability of cancelling the movement varied across sessions. The magnitude of the modulation increased significantly with the probability of failing to cancel the planned movement in the trials during which each neuron was recorded ($r^2 = 0.24$; $t = 5.6$; d.f. = 101; $P < 0.001$) (Fig. 3b). Thus, the strength of the neural signal in the SEF associated with successful cancelling of planned movements was related not to the external variable of stop-signal delay but instead to an internal variable related to performance.

During the countermanding task, gaze-shifting and gaze-holding neurons are activated concurrently when movements are cancelled¹⁴. Because they are mutually incompatible, co-activation of the gaze-holding and gaze-shifting systems engenders a conflict in processing

that is proportional to the magnitude of co-activation^{15,16}. The probability of cancelling a planned eye movement is dictated by the balance of activation of gaze-holding and gaze-shifting neurons because movements are cancelled only if the magnitude of gaze-holding activation exceeds the magnitude of gaze-shifting activation. Thus, the probability of failing to cancel increases as gaze-shifting activation grows. Accordingly, as the probability of failing to cancel increases, the combined magnitude of gaze-shifting and gaze-holding activation sufficient to cancel a planned movement will be higher, thereby generating more conflict. The relationship we observed in SEF neurons of the second type (Fig. 3b) corresponds to this measure of conflict.

Trials in which movements are not cancelled despite the stop signal provide a critical test of this interpretation. Under the particular conditions of this eye movement countermanding task, when planned movements were not cancelled, gaze-holding

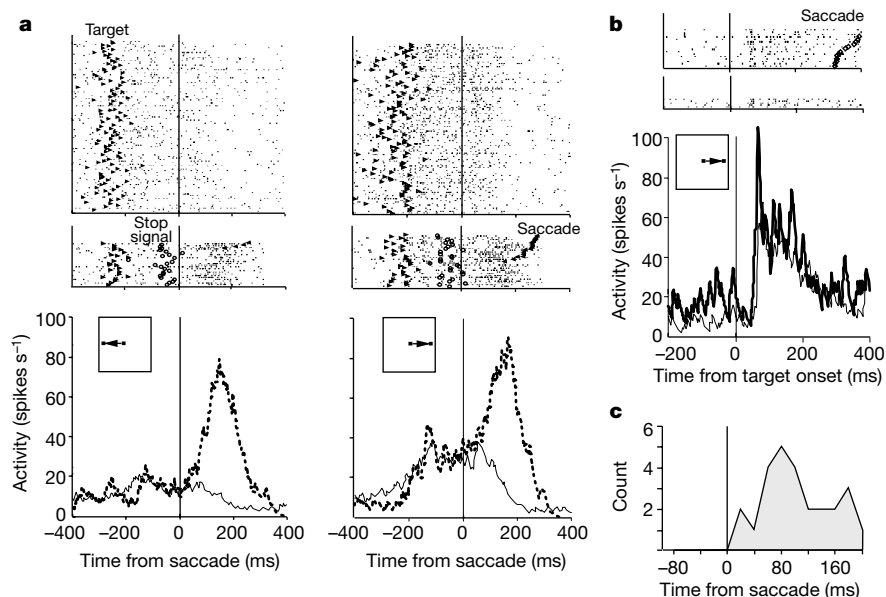


Figure 1 Putative error signal. **a**, Neural activity in no-stop-signal (top) and non-cancelled stop-signal (middle) trials, aligned on initiation of leftward and rightward eye movements with times of target, stop signal, and occasional gaze shift away from the target. Bottom, average discharge rate in no-stop-signal (solid lines) and non-cancelled (dotted lines)

trials. **b**, Activity in no-stop-signal (top) and cancelled stop-signal trials (middle) aligned on presentation of target with times of eye movement in no-stop-signal trials. Bottom, average discharge rate in no-stop-signal (thin solid lines) and cancelled (thick lines) trials. **c**, Latencies of modulation after errors.

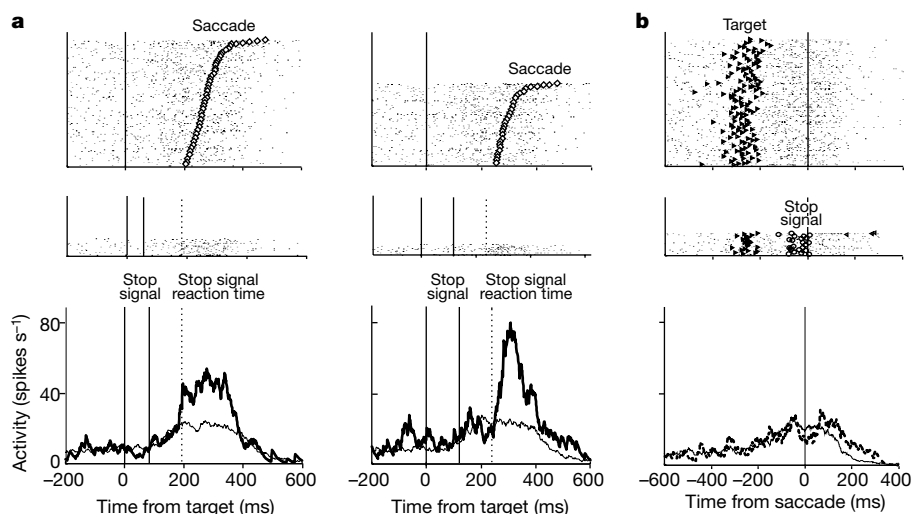


Figure 2 Putative conflict signal. **a**, Activity in no-stop-signal (top) and cancelled stop-signal (middle) trials with stop-signal delays of 93 ms (left) and 144 ms (right)—yielding 3% and 27% non-cancelled errors—with times of stop signal, stop-signal reaction time and eye movement indicated. Bottom: average discharge rate in no-stop-signal (thin lines)

and cancelled (thick lines) trials. **b**, Activity in no-stop-signal (top) and non-cancelled stop-signal (middle) trials with times of target presentation, stop signal and gaze shift. Bottom: average discharge rate in no-stop-signal (thin line) and non-cancelled (thick line) trials.

neurons were not activated¹⁴. Hence, by definition there is no conflict of processing in non-cancelled trials. If the second type of neural modulation in SEF represents conflict in processing, then it should not occur on non-cancelled trials. To test this prediction, the magnitude of modulation in cancelled and non-cancelled trials was quantified for each neuron. Neurons that exhibited modulation in cancelled trials most commonly were not modulated in non-cancelled trials and vice versa ($\chi^2 = 762.64$; d.f. = 20; $P < 0.0001$; Fig. 3 in Supplementary Information). Thus, the data support the hypothesis that the second type of neuron in the SEF signals processing conflict.

The third type of activity in the SEF required another interpretation (Fig. 4). The countermanding task dissociates behaviour from reinforcement. Identical actions (saccades to the target) yield different outcomes (successful no-stop-signal trials or unsuccessful non-cancelled trials). Conversely, different actions (saccades when no stop signal was presented or holding fixation when the stop signal was presented) lead to the same outcome (reinforcement). These conditions permit the distinction between neuronal signals

related to producing the behavioural response from those related to the reinforcement of that response. Although we do not have definitive data, orofacial movements provide an unsatisfactory explanation of the third type of modulation for several reasons. First, inspection of the monkeys during recordings revealed no association between the activity of these neurons and movements of the mouth. Second, the time course and level of activation was the same for trials that earned the primary reinforcer plus the secondary reinforcer as it was for trials that earned only the secondary reinforcer. Third, other investigators have reported reward-related activity in the SEF that could be dissociated from activity related to mouth movements¹⁷. Finally, the reinforcement-related signal in the SEF resembles neural activity associated with the receipt of reward recorded in other structures connected with the SEF¹⁸. Thus, neurons of the third type were the functional complement of the putative error-related neurons, signalling the expectation and receipt of reinforcement.

Whereas neural activity in the FEF was sufficient to cancel motor planning¹⁴ or to initiate saccades¹⁹, the present findings indicate markedly different neural modulation in the SEF, despite numerous parallels between the areas⁴. In fact, the SEF is not necessary for producing accurate visually guided saccades²⁰. These observations suggest a new framework for understanding SEF function. While performing the countermanding task, subjects adjust performance across trials, increasing response time following trials with stop signals, for example²¹. Such self-adjustments have inspired the concept of a supervisory control system that monitors and controls the perception and production systems during decision making, error correction, production of responses that are not well-learned and in overcoming habitual responses^{22,23}. The present results indicate that when monkeys must exert control over the initiation of an eye movement, neurons in the SEF may signal the production of an error, the anticipation of reinforcement or the presence of processing conflict. Indeed, modulated SEF activity is observed in other tasks that require suppressing prepotent responses to produce arbitrary conditional responses^{24–26}. What role might the SEF play in the countermanding task? The likelihood of cancelling a movement if the stop signal happens is increased if the reaction time is

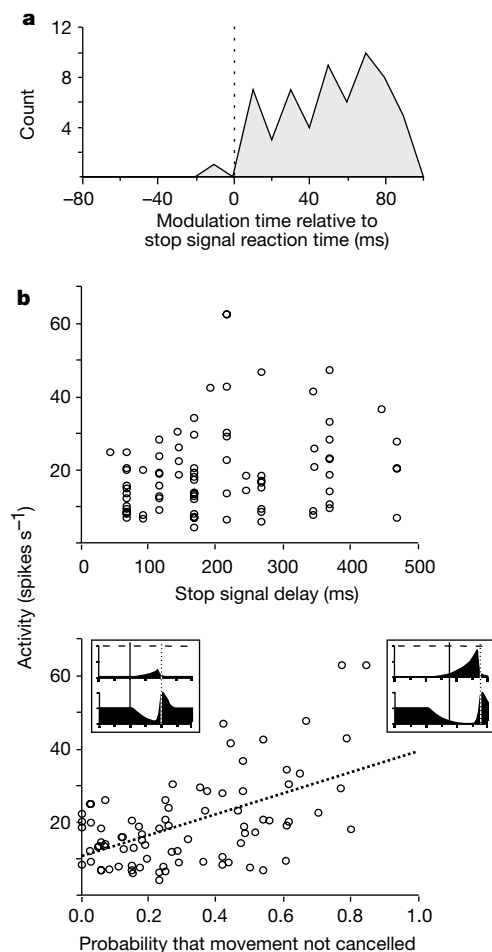


Figure 3 Population data of putative conflict signal. **a**, Latencies of modulation after cancelled movements in relation to stop-signal reaction time. **b**, Magnitude of modulation in cancelled trials plotted against stop-signal delay (top) and failure probability (bottom). Each neuron contributed a point for each stop-signal delay with sufficient data. Dotted line plots regression. Inset panels illustrate state of activation of gaze-shifting neurons (top) and gaze-holding neurons (bottom). Conflict corresponds to the magnitude of co-activation of mutually incompatible processes. On trials with low failure probability (left inset), gaze-shifting activation increases only slightly before the gaze-holding activation cancels movement preparation. Thus, the co-activation of the opposing processes is smaller. On trials with higher failure probability (right inset), gaze-shifting activation increases more before gaze-holding activity cancels movement preparation. Consequently, the co-activation of the opposing processes is greater.

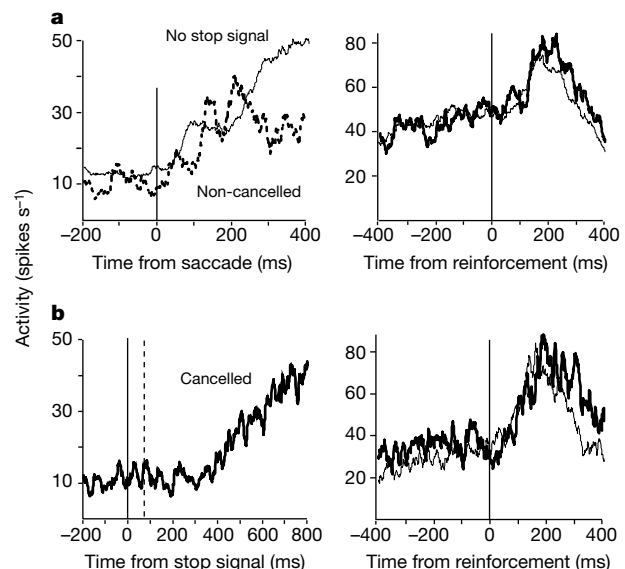


Figure 4 Putative reinforcement signal. **a**, Left, activation grew after successful no-stop-signal trials (thin line) but was reduced in non-cancelled trials (thick dotted line). Right, activation was increased while the monkey awaited reinforcement, and peaked after delivery of primary plus secondary (thick line) or only secondary (thin line) reinforcement. **b**, In cancelled trials, activation grew after the stop-signal reaction time (left) and showed the same increase before reinforcement and the same peak after delivery of primary plus secondary reinforcement (thick line) or only secondary reinforcement (thin line).

longer. Recent findings indicate that activation of the SEF can influence the excitability of gaze centres; when monkeys suppress a reflexive saccade to produce an antisaccade, neurons in the SEF show increased activity²⁴ while neurons in FEF²⁷ exhibit reduced activity. Also, electrical stimulation of the SEF inhibits neuronal activity in the FEF²⁸ and can delay movements^{29,30}. Thus, diverse observations about SEF function might be accommodated by the hypothesis that the SEF functions as a node in the brain's supervisory control system. □

Methods

Two male macaque monkeys (*Macaca mulatta*, *Macaca radiata*) were prepared for training and physiological recording using aseptic procedures under isoflurane anaesthesia. The experimental protocol conformed to United States Public Health Service guidelines and was approved by the Vanderbilt Animal Care Committee. A PDP-11/83 presented stimuli and collected eye position, spike and event data.

The application of the eye movement countermanding task in neurophysiological experiments has been described¹⁴. After a central spot was fixated, it disappeared at the same time as a visual target was presented either in the most sensitive zone of a neuron's response field or in the opposite hemifield at the same eccentricity. On a fraction of trials after a delay, referred to as the stop-signal delay, the fixation spot reappeared, instructing monkeys to withhold the movement ('stop-signal trials'). During the trials in which the stop signal was not presented ('no-stop-signal trials') monkeys were rewarded for generating a single saccade to the peripheral target. During stop-signal trials monkeys were rewarded for maintaining fixation on the central spot ('cancelled trials'). If the monkeys generated a saccade to the peripheral target during stop-signal trials ('non-cancelled trials'), no reward was given. On correct trials juice reward was given on a variable ratio schedule coupled with an acoustic secondary reinforcer given on every trial.

Performance in the countermanding task is probabilistic because of the variability in reaction times across trials. The probability of not cancelling the movement increases as the delay between the signal to initiate the movement and the signal to inhibit the movement ('stop-signal delay') increases. Stop-signal delays were varied according to the monkeys' performance, so that at the shortest (longest) stop-signal delay monkeys generally inhibited the movement on more than 85% (less than 15%) of the stop-signal trials. Movements generated with a short latency tend to be initiated before the stop-signal can influence the system. Conversely, movements generated with long latencies tend to be inhibited because there is enough time for the stop signal to influence the system. The time needed to cancel the movement, known as 'stop-signal reaction time', can be estimated from a simple race model; this model determines the response time on no-signal trials that corresponds to the probability of cancelling a movement at each stop-signal delay. The mean stop-signal reaction time calculated from the behavioural data collected while recording from SEF neurons was 100 ms (A, 104 ms; H, 95 ms).

Neural activity was compared between different types of trials using average activation functions constructed by convolving spike trains with a combination of growth and decay exponential functions that resembled a postsynaptic potential. Criteria for a significant difference in activity between either cancelled or non-cancelled trials and the appropriate latency-matched no-signal trials was that the difference in average firing rate exceed by 2 standard deviations (s.d.) the mean difference in activity during the 600-ms interval before target presentation, provided that the difference reached 6 s.d. and remained above the 2 s.d. threshold for 50 ms. The time interval between the beginning of differential activity and the stop signal reaction time was then determined. The magnitude of modulation in cancelled trials was measured as the time-averaged difference in discharge rate between the activity on cancelled and latency-matched no signal trials.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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High constitutive activity of native H₃ receptors regulates histamine neurons in brain

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Some G-protein-coupled receptors display 'constitutive activity', that is, spontaneous activity in the absence of agonist^{1–4}. This means that a proportion of the receptor population spontaneously undergoes an allosteric transition, leading to a conformation that can bind G proteins⁵. The process has been shown to occur with recombinant receptors expressed at high density, and/or mutated, but also non-mutated recombinant receptors expressed at physiological concentrations^{5–7}. Transgenic mice that express a constitutively active mutant of the β_2 -adrenergic receptor display

cardiac anomalies⁸; and spontaneous receptor mutations leading to constitutive activity are at the origin of some human diseases^{9,10}. Nevertheless, this process has not previously been found to occur in animals expressing normal levels of receptor^{3,4}. Here we show that two isoforms of the recombinant rat H₃ receptor^{11,12} display high constitutive activity. Using drugs that abrogate this activity ('inverse agonists') and a drug that opposes both agonists and inverse agonists ('neutral antagonist'), we show that constitutive activity of native H₃ receptors is present in rodent brain and that it controls histaminergic neuron activity *in vivo*. Inverse agonists may therefore find therapeutic applications, even in the case of diseases involving non-mutated receptors expressed at normal levels.

Starting from the sequence of the human H₃ receptor¹³, we screened a complementary DNA library from rat striatum and

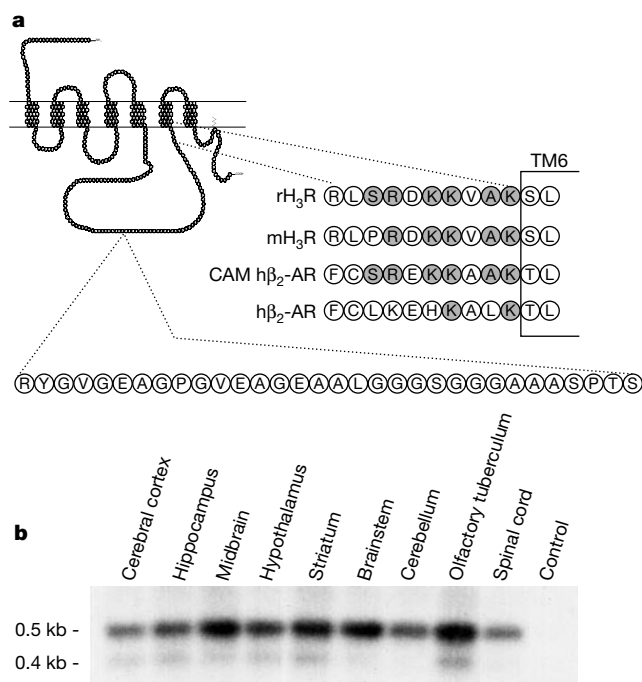


Figure 1 Two rat H₃-receptor isoforms and their expression in rat brain regions. **a**, Putative seven-transmembrane topography of H_{3S} and H_{3L} alternatively spliced variants differing by a 32-amino-acid insertion in the i3 loop. This loop C-terminal sequence is compared with that of the mouse (mH_{3R}), the native human β₂-adrenergic receptor (hβ₂-AR) and a constitutively active mutant (CAM hβ₂-AR) (ref. 20). **b**, RT-PCR analysis of H_{3S} and H_{3L}-receptor RNAs using primers flanking the spliced region.

Table 1 Potencies of H₃-receptor ligands in CHO(H_{3S}) and CHO(H_{3L}) cells

Agents	^{[3]H} histamine release K _i or EC ₅₀ (nM)	^{[3]H} arachidonic acid release K _i or EC ₅₀ (nM)	
		H _{3S}	H _{3L}
Agonists			
Histamine	200 ± 50*	110 ± 32	141 ± 39
Imetit	1.0 ± 0.3†	5 ± 2	0.4 ± 0.2
Inverse agonists§			
Thioperamide	2.2 ± 0.6	0.3 ± 0.1	0.05 ± 0.03
Ciproxifan	0.5 ± 0.1‡	0.07 ± 0.03	0.11 ± 0.06
FUB 465	580 ± 230	18 ± 6	10 ± 5
Antagonist			
Proxyfan	19 ± 6	5.0 ± 1.7	6.3 ± 2.3

The potencies of H₃-receptor ligands were compared for both [³H]histamine release from depolarized synaptosomes and A23187-evoked [³H]arachidonic acid release from CHO(H_{3S}) and CHO(H_{3L}) cells.

* Data from ref. 26.

† Data from ref. 24.

‡ Data from ref. 25.

§ Values represent the EC₅₀ for [³H]arachidonic acid release and the K_i for [³H]histamine release from rat cortical synaptosomes when opposed to histamine in 30 mM K⁺ medium.

isolated two full-length complementary DNAs encoding 445- and 413-amino-acid sequences (Fig. 1a) that we termed H_{3L} and H_{3S}, in analogy with the corresponding H₃-receptor variants in guinea-pig¹⁴. The existence of these variants results from splicing at a level corresponding to the mid-portion of the third intracytoplasmic loop (i3) of the H₃ receptor. Alternative splicing of the dopamine D₂ receptor^{15,16} at almost the same level leads to two isoforms with similar pharmacology and only limited differences in G-protein-coupling efficiency. In the case of the H₃ receptor, comparative binding studies on different tissues and the multiphasic competition curves observed with some antagonists has suggested the existence of distinct receptor subtypes^{17–19}. The existence of isoforms derived from the same gene with limited pharmacological differences (Table 1) may account partly for these observations, although our polymerase chain reaction (PCR) study shows that the H_{3L} receptor is largely predominant in all tissues (Fig. 1b).

The carboxy terminus of i3 has a stretch of eight amino acids that are highly similar to the corresponding sequence of a mutated human β₂-adrenergic receptor in which the mutation confers a constitutive activity (CAM hβ₂-AR in Fig. 1a) that is absent in the native receptor²⁰. Thus, among these eight amino acids, six (five in the mouse) are identical in the rat H₃ receptor and in the mutated β₂-adrenergic receptor, whereas the other two amino acids are conserved. Furthermore, this region is also critical for constitutive activity in other native or mutated heptahelical receptors^{5,9,21}.

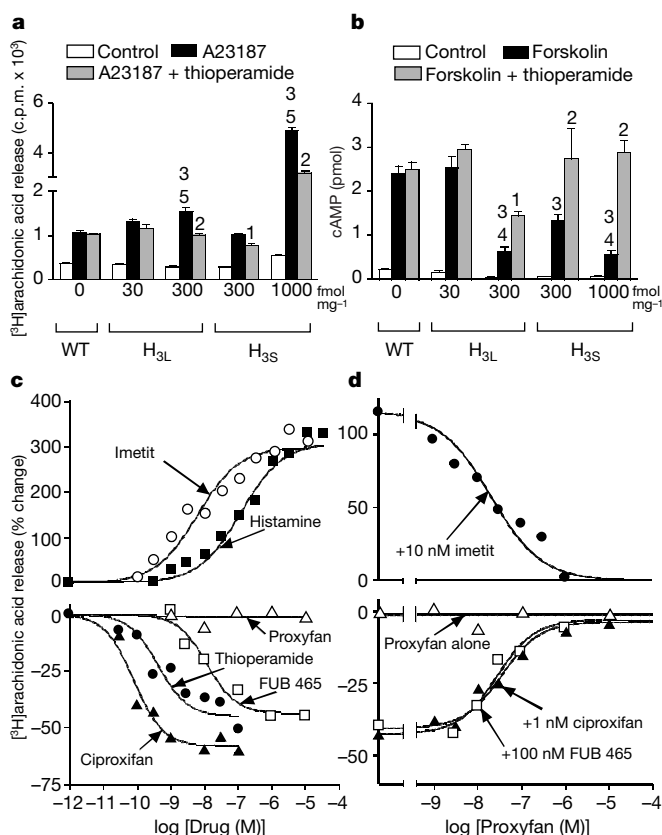


Figure 2 Constitutive activity and pharmacology of H_{3S} or H_{3L} receptors expressed in CHO cells. Effects of thioperamide on A23187-evoked [³H]arachidonic acid release (**a**) or forskolin-evoked cAMP accumulation (**b**) in CHO cells expressing various densities of the two isoforms. Results are means ± s.e.m. of 3–10 determinations in an experiment replicated with similar data. P < 0.05 (1), P < 0.001 (2) versus A23187 or forskolin alone; P < 0.001 (3) versus wild-type cells; P < 0.01 (4), P < 0.001 (5) versus CHO(H_{3S}) cells expressing 300 fmol per mg protein. **c**, **d**, Effects of H₃-receptor ligands on A23187-evoked [³H]arachidonic acid release from CHO(H_{3S}) cells expressing 500 fmol per mg protein. Results are expressed as the per cent change compared with A23187-evoked release (1,308 ± 22 c.p.m. per well).

We have therefore assessed the constitutive activity of the H_{3L} and H_{3S} isoforms expressed in Chinese hamster ovary (CHO) cells at low, medium and high densities: 30–80, 300–500 and ~1,000 fmol per mg protein as determined by [125 I]iodoproxyfan assay²², respectively. The coupling changes associated with receptor expression were evaluated in two signalling pathways activated by histamine and involving G_i/G_o proteins: adenylyl cyclase inhibition and phospholipase A_2 activation. In both pathways, constitutive activity of both the H_{3L} and H_{3S} receptors was clearly evidenced. In addition, [3 H]arachidonic acid release evoked by the Ca^{2+} -ionophore A23187 was enhanced, whereas forskolin-induced cyclic AMP accumulation was reduced (in both cases in total absence of histamine) when the receptor density was enhanced; these changes were largely reversed in the presence of thioperamide, a compound so far considered as the prototypical H_3 -receptor antagonist¹², but functioning here, as predicted by binding data²³, as an extremely potent inverse agonist (Fig. 2).

Constitutive activity was slightly more pronounced with the H_{3L}

isoform: there was a tendency for spontaneous activity and a response to thioperamide at low expression levels of H_{3L} (effects that became significant at 80 fmol per mg protein; data not shown), and, at intermediate levels, changes were more marked than with the H_{3S} isoform (Fig. 2a, b); however, the difference between the isoforms was modest as also shown by the limited difference in potency of the agonist imetit²⁴ (and the similar potency of histamine). A primary characteristic of the active conformation of G-protein-coupled receptors is their higher affinity for agonists³.

These observations suggested that constitutive H_3 -receptor activity was likely to occur in brain where the H_{3L} isoform predominates, and where the density of [125 I]iodoproxyfan-binding sites²² is presumably more than 500 fmol per mg protein in cells expressing the H_3 receptor (assuming that these cells represent less than 20% of the total). To assess this possibility, we needed to identify ligands displaying well-defined agonist, inverse agonist and neutral antagonist properties in cell lines, and then to determine the effects of these probes at the native cerebral receptor. Neutral antagonists are not

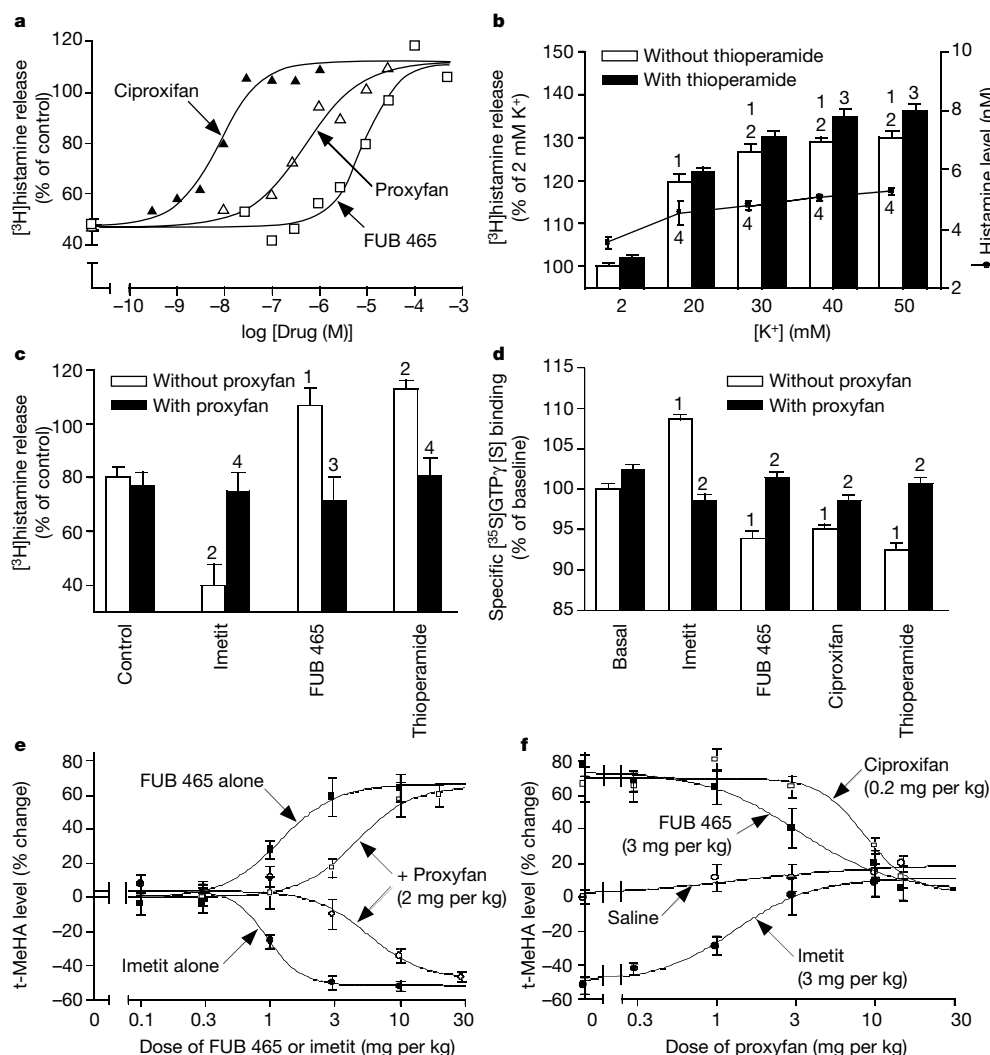


Figure 3 Effects of H_3 -receptor ligands on responses mediated by native H_3 receptors in rodent brain. **a**, Reversal of histamine-induced inhibition of [3 H]histamine release from rat synaptosomes depolarized by 30 mM K^+ . Means from two separate experiments with quadruplicate determinations in each. **b**, Effect of thioperamide on [3 H]histamine release from mouse synaptosomes depolarized by increasing K^+ concentrations. Levels of endogenous histamine released in the medium by K^+ were determined. Results are means $\pm$ s.e.m. of 13–23 determinations from three separate experiments. $P < 0.001$ (1) versus 2 mM K^+ ; $P < 0.01$ (2) versus 20 mM K^+ ; $P < 0.05$ (3) versus without thioperamide; $P < 0.05$ (4) versus 2 mM K^+ . **c**, Effects of H_3 -receptor ligands on

[3 H]histamine release induced by 55 mM K^+ from mouse synaptosomes in the presence or absence of proxyfan. Means $\pm$ s.e.m. of 12–40 determinations from four separate experiments. $P < 0.01$ (1), $P < 0.001$ (2) versus control; $P < 0.01$ (3), $P < 0.001$ (4) versus without proxyfan. **d**, Effects of H_3 -receptor ligands on [35 S]GTP- γ [S] binding to mouse cerebral cortical membranes in the presence or absence of proxyfan. Results are means $\pm$ s.e.m. of 9–24 determinations from four separate experiments. $P < 0.001$ (1) versus basal; $P < 0.01$ (2) versus without proxyfan. **e**, **f**, Changes in brain t-MeHA levels in mice receiving H_3 -receptor ligands p.o. Means $\pm$ s.e.m. of 8–16 values.

easily identified because, theoretically, they correspond to ligands displaying equal affinity for the active and inactive receptor conformations, a condition obviously difficult to achieve^{1,4}. We found that a large number of tested antagonists (defined by their ability to block the histamine response at the autoreceptor inhibiting [³H]histamine release from synaptosomes) behaved as potent inverse agonists: they decreased [³H]arachidonic acid release from CHO cells at concentrations up to two orders of magnitude less than those required to antagonize histamine at the H₃ autoreceptor in synaptosomes. This included FUB 465 (ethyl-3-(1*H*-imidazol-4-yl)propyl ether), an antagonist with only micromolar affinity at the synaptosomal autoreceptor (Fig. 3a) and with inverse agonist activity with an effective concentration for half-maximal response (EC₅₀) of ~10 nM in CHO(H₃) cells (Fig. 2c).

In contrast, we identified proxyfan (3-(1*H*-imidazol-4-yl)propyl-phenylmethyl ether) as a potent (inhibition constant (*K*_i) ≈ 10 nM) neutral antagonist: first, it inhibited the effects of histamine at the synaptosomal H₃ autoreceptor (Fig. 3a); second, in CHO(H₃) cells with moderate expression, it inhibited those of the agonist imetit and those of the inverse agonists ciproxifan²⁵ and FUB 465, without affecting [³H]arachidonic acid release alone, even at a 10 μM concentration (Fig. 2d). As expected¹, however, the pharmacological profile of proxyfan depended on the test system; that is, it depended on the equilibrium between the active and inactive conformations of the receptor and/or the stoichiometric ratio of the receptor to the various G proteins. Proxyfan displayed partial inverse agonism on [³H]arachidonic acid release in CHO cells with high expression and partial agonism when cAMP was evaluated (data not shown).

Using these various probes, we could determine the constitutive activity of the H₃ autoreceptor controlling [³H]histamine release in cortical synaptosomes submitted to a strong depolarizing stimulus (40–55 mM K⁺) in the mouse (Fig. 3b, c) or rat (data not shown). Both thioperamide and FUB 465 behaved as inverse agonists, enhancing significantly the amine release, release being on the contrary reduced by the agonist imetit. Proxyfan, which alone did not affect [³H]histamine release, blocked the opposite effects of either thioperamide and FUB 465 or imetit, therefore acting again as a neutral antagonist (Fig. 3c). These responses could not involve endogenous histamine, and the endogenous amine level, even in the 55 mM K⁺ medium, was two orders of magnitude less than its EC₅₀ value as an agonist²⁶ (5.3 ± 0.2 nM versus 200 ± 50 nM). Therefore blockade of the H₃-autoreceptor stimulation by endogenous histamine does not significantly contribute to the releasing effect of drugs like thioperamide (as we had originally proposed^{11,12}). This is also shown by (1) the lack of releasing effect of proxyfan, a potent neutral antagonist, (2) the potent releasing effect of FUB 465, a potent inverse agonist but weak neutral antagonist, and (3) the evidence for constitutive activity in cerebral membranes devoid of histamine using a [³⁵S]GTPγS-binding test.

In agreement, binding of the guanylnucleotide analogue [³⁵S]GTPγS to mouse (or rat; data not shown) cerebral membranes demonstrated the coupling of the native H₃ receptor with G proteins²⁷, in other words, constitutive activity (Fig. 3d). Thus, specific [³⁵S]GTPγS binding was reduced significantly by FUB 465, ciproxifan or thioperamide, which were acting as inverse agonists as their effects were blocked by 1 μM proxyfan. Proxyfan also blocked the increase in binding elicited by imetit, but did not itself significantly affect binding and was therefore acting again as a neutral antagonist in this test system. In contrast with this pattern, yohimbine, an inverse agonist at overexpressed or mutated α₂-adrenergic receptors, did not decrease [³⁵S]GTPγS binding to cerebral membranes, indicating that constitutive receptor activity is not an inevitable consequence of the experimental conditions required to evaluate the binding, as previously proposed¹.

Moreover, all three inverse agonists markedly enhanced cerebral histamine neuron activity *in vivo*, increasing the levels of the histamine metabolite *tele*-methylhistamine (t-MeHA), a reliable

marker of this activity²⁸ (Fig. 3e, f), by ~80% at maximum, and increasing histamine turnover as evaluated by the pargylin-induced t-MeHA accumulation in brain (not shown). This effect reflects an inverse agonist rather than the antagonist activity (towards endogenous histamine) of these ligands, as has been assumed so far^{12,28}. In agreement, the effect of FUB 465 obtained at a low dose (half-maximal effective dose (ED₅₀) ≈ 1 mg per kg per os, p.o.) was more consistent with its nanomolar potency as an inverse agonist than its micromolar potency as an antagonist. This situation is not restricted to FUB 465, and the potency of a large series of ligands in enhancing t-MeHA level in brain is not well correlated with their antagonist potency^{29,30}, but more so with their inverse agonist potency (X.L., S.M., C.R.G., W.S., J.-C.S. & J.M.A.; manuscript in preparation). Moreover, the increases in t-MeHA level by FUB 465 and ciproxifan were competitively antagonized by proxyfan given at doses of ~2 mg per kg which also blocked the decrease in t-MeHA level induced by the agonist imetit. At these doses, proxyfan administered alone failed to affect significantly levels of t-MeHA, indicating that it was acting as a neutral antagonist *in vivo* on a system regulated by H₃ receptors displaying constitutive activity. The small but significant increase (by ~20%) observed with proxyfan in doses above 10 mg per kg might reflect its antagonist activity towards endogenous histamine.

Activation of histaminergic neurons, which promotes arousal and attention, and improves learning in normal animals, has been proposed as a symptomatic therapeutic approach in human attentional and ageing disorders, such as attention-deficit hyperactivity disorders and Alzheimer's disease²⁵. Our observations indicate that such an effect is more likely to be obtained with H₃-receptor inverse agonists rather than with neutral antagonists, as has been assumed so far. □

Methods

Cloning of rat H₃ receptor cDNA isoforms

A rat striatal cDNA library (4 × 10⁶ phages; Stratagene) was screened at high stringency with a ³²P-labelled fragment (607 base pairs) obtained by PCR with reverse transcription (RT-PCR) of total RNAs from rat cerebral cortex using primers 1 and 2 (ref. 13). These primers are based on the sequence of the third transmembrane domain and the third intracellular loop of the human H₃ receptor, respectively. Bluescript KS(+) plasmids were recovered from 60 positive clones and their cDNA inserts sequenced. The sequence of the rat H_{3L} and H_{3S}-receptors has been deposited in GenBank (accession numbers AY009370 and AY009371). The full-length mouse H₃-receptor cDNA was obtained by RT-PCR.

RT-PCR analysis

RNAs from various rat brain regions, rat spinal cord (10 μg) and mouse striata (2 μg) were used for first strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase (50 units, Roche) and 0.19 μM random primer p(DN)₆ (Roche). These templates were amplified for 30 cycles (94 °C for 30 s and 68 °C for 10 min) using gene Amp XL PCR kit (Perkin-Elmer), and either primers 3 and 4 corresponding to nucleotides 494–522 and 932–961 of the rat H_{3L}-receptor sequence for amplification in rat, or primers 5 and 6 corresponding to nucleotides 1–33 of the rat H₃-receptor coding sequence and nucleotides 1,601–1,637 of the human H₃-receptor sequence¹³ for amplification in mouse. Rat PCR products were electrophoresed, blotted overnight onto nylon membranes, and hybridized with the ³²P-labelled 607-base-pair cDNA probe described above. Mouse PCR products were subcloned into pGEM-T Easy plasmid (Promega) and sequenced.

Stable transfection of CHO-K1 cells

The cDNA inserts corresponding to the full-length coding sequence of the H_{3S} and H_{3L} isoforms were ligated into the mammalian expression vector pCIneo (Promega). CHO-K1 cells were transfected with SuperFect reagent (Qiagen). Stable transfectants were selected with 2 mg ml⁻¹ G418 and tested for [¹²⁵I]iodoproxyfan binding²². Several clones, named CHO(H_{3S}) and CHO(H_{3L}), expressing various receptor densities, were selected for further characterization and maintained in the presence of 1 mg ml⁻¹ G418.

[¹²⁵I]iodoproxyfan-binding assay

CHO (H_{3L} or H_{3S}) cells were washed and homogenized (Polytron) in ice-cold binding buffer (Na₂HPO₄/KH₂PO₄ 50 mM, pH 6.8), and binding assays were done as described²².

cAMP accumulation

CHO (H_{3L} or H_{3S}) cells (96-well plates) were incubated for 10 min at 37 °C with 3 μM forskolin and, when required, 1 μM thioperamide in DMEM/Nut mix F-12 containing 100 μM isobutylmethylxanthine. cAMP was extracted and measured by

radioimmunoassay (NEN Life Science Products). We carried out statistical evaluation of the results by analysis of variance (ANOVA) followed by Newman–Keuls test.

³H]arachidonic acid release

CHO (H₃L or H₃S) cells (24-well plates) were incubated for 2 h at 37 °C with 0.5 µCi of [³H]arachidonic acid in DMEM/Nut mix F12 containing 0.2% bovine serum albumin. After washings, cells were incubated for 30 min with 2 µM A23187 and, when required, the H₃-receptor ligands and [³H]arachidonic acid release was determined by liquid scintillation counting. We carried out statistical evaluation of the results by ANOVA followed by Newman–Keuls test.

³H]histamine release from synaptosomes

After a 30-min pre-incubation of mouse or rat cortical synaptosomes with [³H]-L-histidine, [³H]histamine release induced by potassium was evaluated²⁶. Total [³H]histamine initially present in synaptosomes represented about 3,500 d.p.m. per mg protein. For reversal of histamine-induced inhibition of [³H]histamine release, drugs in increasing concentrations were opposed to 1 µM histamine in the presence of 30 mM K⁺. The effect of thioperamide (100 nM) was studied on [³H]histamine release induced by 20–55 mM K⁺. The effects of various H₃-receptor ligands (100 nM) were studied on [³H]histamine release induced by 55 mM K⁺ in the presence or absence of 10 µM proxyfan. Statistical evaluation of the results was performed using ANOVA followed by Newman–Keuls test.

Histamine and [³⁵S]GTPγS-binding assays

We measured histamine in the medium of depolarized mouse cortical synaptosomes and of cell cultures using an enzymoimmunoassay (Immunotech, Marseille, France). For [³⁵S]GTPγS binding assays, mouse or rat cerebral cortical membranes were pretreated with adenosine deaminase (1 U ml⁻¹) and incubated as described²⁷ for 30 min at 25 °C with 0.1 nM [³⁵S]GTPγS and various H₃-receptor ligands (10 nM) in the presence or absence of 1 µM proxyfan. Statistical evaluation of the results was performed using ANOVA followed by Newman–Keuls test.

Assay of tele-methylhistamine (t-MeHA) in brain

Drugs were administered p.o. to male Swiss mice (Iffa-Credo, L'Arbresle, France) and brain t-MeHA levels were determined after 90 min by enzymoimmunoassay²⁴. Results are expressed as per cent change compared with values obtained in vehicle-treated mice (119 ± 4 ng per g).

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Attenuation of FGF signalling in mouse β-cells leads to diabetes

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Fibroblast growth factor (FGF) signalling has been implicated in patterning, proliferation and cell differentiation in many organs, including the developing pancreas^{1,2}. Here we show that the FGF receptors (FGFRs) 1 and 2, together with the ligands FGF1, FGF2, FGF4, FGF5, FGF7 and FGF10, are expressed in adult mouse β-cells, indicating that FGF signalling may have a role in differentiated β-cells. When we perturbed signalling by expressing dominant-negative forms of the receptors, FGFR1c and FGFR2b, in the pancreas, we found that mice with attenuated FGFR1c signalling, but not those with reduced FGFR2b signalling, develop diabetes with age and exhibit a decreased number of β-cells, impaired expression of glucose transporter 2 and increased proinsulin content in β-cells owing to impaired expression of prohormone convertases 1/3 and 2. These defects are all characteristic of patients with type-2 diabetes. Mutations in the homeobox gene *Ipfl/Pdx1* are linked to diabetes in both mouse and human. We also show that *Ipfl/Pdx1* is required for the expression of FGFR1 signalling components in β-cells, indicating that *Ipfl/Pdx1* acts upstream of FGFR1 signalling in β-cells to maintain proper glucose sensing, insulin processing and glucose homeostasis.

Pancreatic β-cell dysfunction is a critical component in the development of type-2 diabetes. The disease results in part from an inability of β-cells to produce and secrete sufficient amounts of active insulin in response to an increased demand for insulin^{3,4}. To decipher the molecular machinery of β-cell function, we first investigated whether extrinsic factors, such as FGF signalling factors, are involved in the differentiation of pancreatic β-cells. We analysed the expression of FGFR1 and FGFR2 in the adult mouse pancreas. FGFR1 and FGFR2 were both expressed predominantly in β-cells, with no expression observed in glucagon-producing α-cells (Fig. 1a, d). A low level of FGFR1 expression was also observed in cells of the exocrine pancreas, whereas no expression

of FGFR2 was observed in these cells (data not shown). Six ligands, FGF1, FGF2, FGF4, FGF5, FGF7 and FGF10, were also expressed in β -cells, with no expression observed in α -cells (Fig. 1b, c, e, f; and data not shown). Together, these expression patterns suggest that an autocrine loop of FGF signalling may be involved in terminal differentiation and/or maturation of β -cells.

Genetic approaches to elucidate the role of FGF signalling in the mouse have been hampered by early embryonic lethality or functional redundancy^{1,2}. A successful alternative approach has been to attenuate FGF signalling through expression of soluble dominant-negative forms of FGFRs that bind to and block the interaction of FGFs with the normal receptor^{3,6}. To examine FGF signalling in the genesis and function of β -cells, we generated two transgenic mouse

lines expressing a dominant-negative version of the FGFR1 and FGFR2 isoforms, FGFR1c⁶ and FGFR2b^{7,8}, in the developing and adult pancreas using the *Ipfl/Pdx1* promoter⁹. The transgenes were expressed at high levels in β -cells and at lower levels in the exocrine cells of the pancreas of transgenic mice (Fig. 1g–l; and data not shown). We designated these two transgenic mice as FRID1 (for FGFR1c/Ipfl diabetic 1) and FRIND2 (for FGFR2b/Ipfl non-diabetic 2).

FRID1 and FRIND2 neonates were indistinguishable from their littermates with an apparently well-developed pancreas (Fig. 1g–o; and data not shown), indicating that signalling through FGFR1c or FGFR2b under these conditions is not pivotal for growth, morphogenesis or differentiation of the pancreas. The FRID1 mice grew normally, were lean and appeared healthy until about 15 weeks, when elevated non-fasting urine glucose concentrations greater than 2% (> 11.1 mM; see Methods) were observed, suggesting a diabetic phenotype. At 3 weeks, FRID1 mice displayed normal fasting blood glucose levels; at 6 weeks, fasting blood glucose levels had increased slightly but were still within the range of normoglycaemia. However, by 9–12 weeks these mice were overt diabetic with urine glucose levels in excess of 2%, and non-fasting and fasting blood glucose levels nearly fourfold higher than those of age-matched wild-type littermates (Table 1). In contrast, FRIND2 mice exhibited normal fed blood glucose levels as compared to age-matched littermates (8.9 ± 0.6 mM, $n = 6$, and 8.7 ± 0.3 mM, $n = 6$, respectively), and did not develop diabetes. These findings show that impairment of FGFR1c signalling in pancreatic β -cells leads to diabetes, indicating that FGFR1c signalling has a crucial role in controlling glucose homeostasis. In contrast, perturbation of FGFR2b signalling does not result in diabetes, indicating that there is a specificity and selectivity by which signalling through FGFR1c in β -cells ensures normoglycaemia.

To analyse in detail the differentiated state of the pancreas in 10-week-old FRID1 and FRIND2 mice, we examined the expression of transcription factors, hormones and enzymes. The transcription factors¹⁰ IPF1/PDX1, ISL1, Nkx6.1, Nkx2.2, the hormones insulin, glucagon, somatostatin, the pancreatic polypeptide, and the exocrine enzymes amylase and carboxypeptidase A were all expressed in FRID1 and FRIND2 mice (Fig. 1g–o; and data not shown). The organization of endocrine cells into islet-like clusters, and of exocrine cells into acinar-like structures also appeared normal (Fig. 1; and data not shown). The islet cells in the FRID1 mice revealed, however, an atypical organization with α -cells scattered throughout the islets (Fig. 1m–o).

In addition, there was a ~25% net decrease in the number of insulin-positive cells ($n = 4$, $P < 0.05$) accompanied by a 25% decrease in total pancreatic insulin content in adult FRID1 mice ($n = 6$, $P < 0.05$). The absolute number of glucagon-positive, somatostatin-positive and pancreatic-polypeptide-positive cells was unchanged (data not shown). These results suggest that the genesis and/or survival of β -cells are partly dependent on FGFR1c signalling. No increased β -cell apoptosis was observed as determined by TdI-mediated dUTP nick end labelling (TUNEL) assay

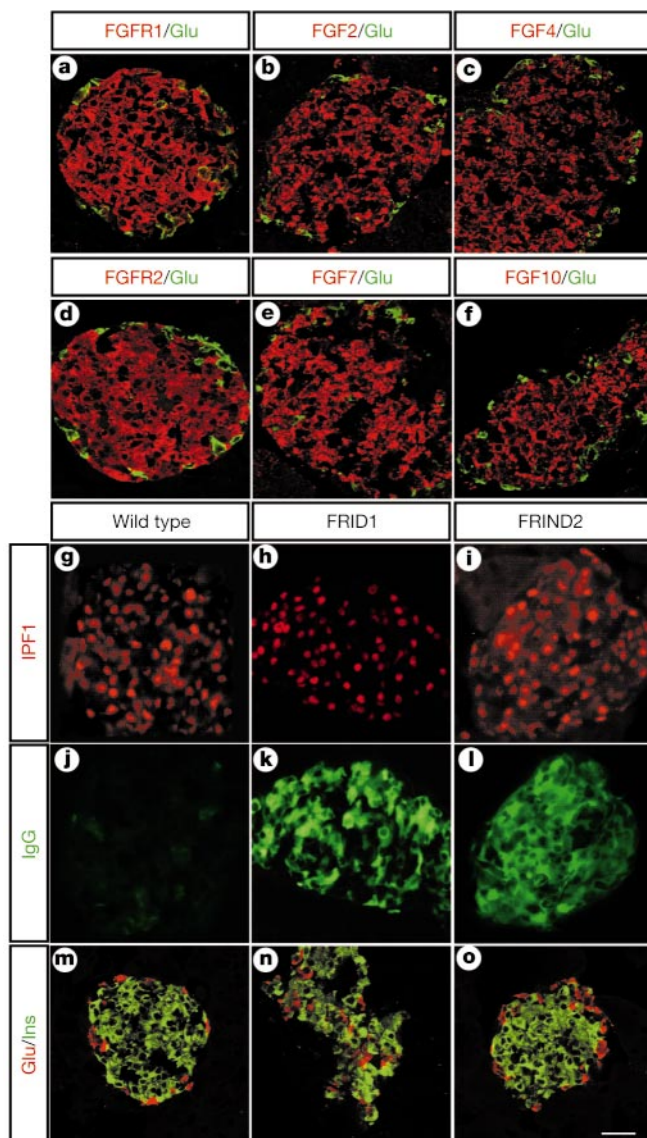


Figure 1 FGF signalling components are expressed in adult β -cells. Confocal microscopy analyses of FGF ligand and receptor expression in adult mouse pancreas showing that the receptors FGFR1 (a) and FGFR2 (d), and the ligands FGF2 (b), FGF4 (c), FGF7 (e) and FGF10 (f) are expressed in β - but not α -cells. Dominant-negative FGFR1c (k) and FGFR2b (l) transgene expression can be detected in IPF1/PDX1-positive cells (g–i) of FRID1 (h, k, n) and FRIND2 (i, l, o) mice by staining for the Fc IgG region (j–l). Confocal microscopy analyses of insulin and glucagon expression (m–o) in 10-week-old wild-type (g, j, m), FRID1 (h, k, n) and FRIND2 (i, l, o) mice reveal an abnormal organization of the islets in FRID1 mice. Images are representative of at least four animals from each group. Scale bars, 20 μ m.

Table 1 FRID1 mice develop diabetes

	Blood glucose levels (mM $\pm$ s.e.m.)	
	Non-fasted	Fasted
Wild type (3 weeks old)	nd	4.4 ± 0.4 ($n = 5$)
FRID1 (3 weeks old)	nd	5.8 ± 0.5 ($n = 7$)
Wild type (6 weeks old)	nd	4.9 ± 0.3 ($n = 5$)
FRID1 (6 weeks old)	nd	6.8 ± 0.8 ($n = 7$)
Wild type (9–12 weeks old)	8.7 ± 0.8 ($n = 5$)	4.0 ± 0.6 ($n = 5$)
FRID1 (9–12 weeks old)	26.4 ± 1.5 ($n = 5$)	15.8 ± 1.3 ($n = 5$)

Blood glucose levels were measured in FRID1 mice and wild-type littermates at the time points shown. At 3 weeks of age FRID1 mice showed non-fasted blood glucose levels within the normal range. Six-week-old FRID1 mice had slightly elevated non-fasted blood glucose levels, towards the upper end of the normal range. Overt diabetes (OD) develops in 9–12-week-old mice in which both non-fasted and fasted blood glucose levels were fourfold higher than in wild-type, age-matched littermates.

(data not shown), suggesting that the decreased number of β -cells in the FRID1 mice is not caused by β -cell death but rather by impaired β -cell differentiation and/or replication. β -cell neogenesis normally proceeds in mice up to ~ 3 weeks of age¹¹. In FRID1 neonatal mice the numbers of β -cells were equivalent to those of wild-type littermates; however, at postnatal day (P)-10 the β -cell number was $\sim 8\%$ less than that of wild-type littermates ($n = 3$), and by P27 $\sim 11\%$ less β -cells ($n = 4$) were observed. These results show a progressive impairment in β -cell expansion in FRID1 mice. Together, these findings provide evidence for a role for FGFR1c signalling in the postnatal expansion of β -cells¹¹.

The 25% decrease in total insulin production appeared unlikely to cause the diabetes observed in the FRID1 mice, indicating that additional β -cell defects contribute to the development of diabetes. To address this issue, we examined the expression of factors critically required to maintain glucose homeostasis. Glut2 is a component of the glucose-sensing machinery in the β -cell¹²,

although a direct role for Glut2 in maintaining normoglycaemia in human β -cells has not been proved. Nevertheless, *Glut2* null mutant mice develop early diabetes¹², and β -cells of *Glut2*^{-/-} mice show a loss of first phase insulin secretion in response to glucose stimulation¹². Specific expression of either Glut2 or Glut1 in β -cells of *Glut2*^{-/-} mice results in a restored normal glucose-stimulated insulin secretion¹³. These findings show that Glut2 is involved in maintaining glucose homeostasis. In the 10-week-old, overt diabetic FRID1 mice, the expression of Glut2 was absent (Fig. 2a, b); however, the expression of glucokinase, another key component of the glucose-sensing machinery¹⁴, appeared indistinguishable from that of wild-type littermates (data not shown). To show that the loss of Glut2 expression was not merely a consequence of the hyperglycaemic state, but instead reflected a direct effect of the impaired FGFR1c signalling, we analysed 5-week-old, prediabetic transgenic mice. These mice also exhibited a reduced level of Glut2 expression as compared with wild-type littermates (data not shown). These results indicate that the reduction of Glut2 expression observed in FRID1 mice is a direct consequence of impaired FGFR1c signalling.

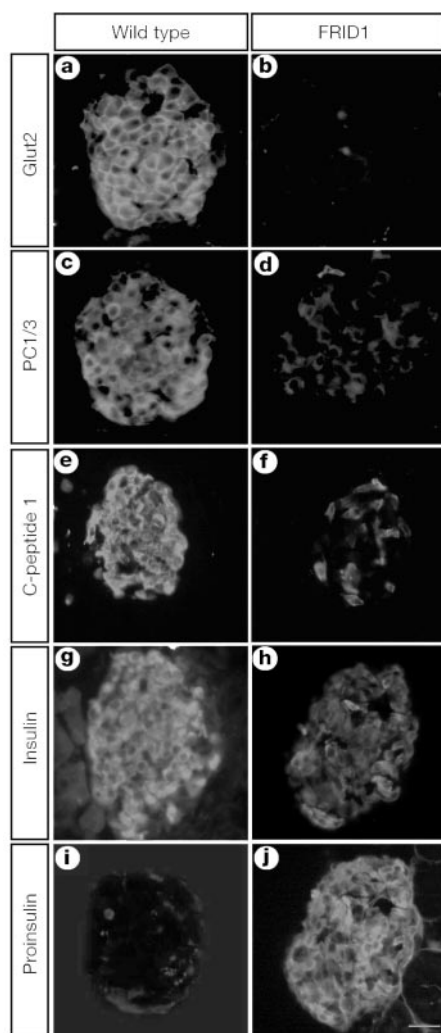


Figure 2 Expression of glucose-sensing and insulin-processing enzymes is impaired in FRID1 mice. Images show Glut2 (a, b), PC1/3 (c, d), C-peptide 1 (e, f), insulin (g, h), and proinsulin (i, j) expression in 10-week-old wild-type (a, c, e, g, i) and FRID1 (b, d, f, h, j) mice. (a–d) Glut2 expression in adult β -cells is lost as a result of dominant-negative FGFR1c expression (a, b) and only a very low, barely detectable level of PC1/3 expression remains in FRID1 mice (c, d). In wild-type pancreas, insulin is present predominantly in its fully processed form (e, g) with virtually no detectable unprocessed proinsulin (f). FRID1 mice display perturbed proinsulin processing, resulting in reduction of fully processed insulin (f, h) with a substantial, readily detectable fraction remaining in the form of proinsulin (j). Images are representative of at least six animals from each group. Scale bars, 20 μ m.

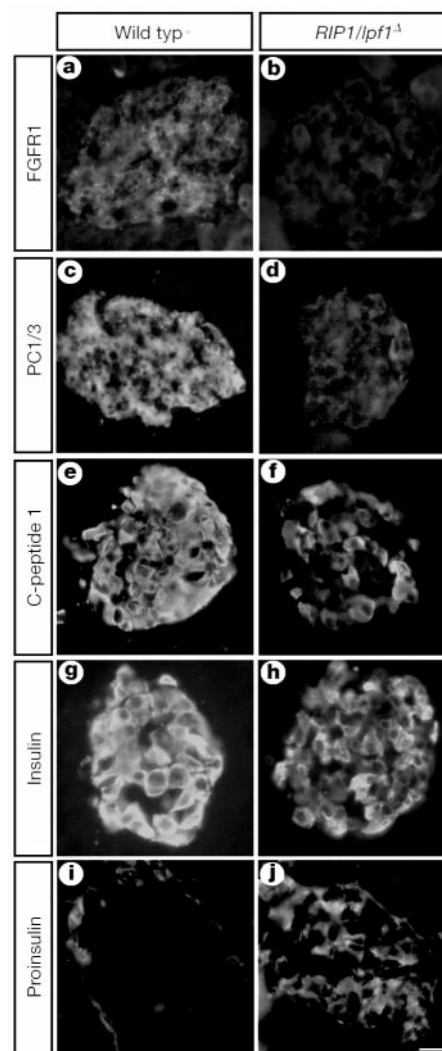


Figure 3 *lpf1/Pdx1* controls several aspects of β -cell identity including FGFR1 expression. Loss of IPF1/PDX1 activity in β -cells²² results in markedly reduced FGFR1 (a, b) and PC1/3 (c, d) expression. The loss of PC1/3 expression results in perturbed proinsulin processing, with a reduction in detectable levels of fully processed insulin, compared with in wild-type littermates (e–h). This is accompanied by increased levels of detectable proinsulin in the β -cells of *Rip1/lpf1*^Δ mice (i, j). Images are representative of at least four animals from each group. Scale bars, 20 μ m.

Although loss of Glut2 expression may be sufficient to cause a diabetic phenotype¹², the severity of the ensuing hyperglycaemia in a short period of time suggested that there might be additional perturbations of β -cell function in FRID1 mice. Type-2 diabetic patients often suffer from hyperproinsulinaemia, suggesting that they have impaired processing of proinsulin to mature active insulin^{15–17}. Hyperproinsulinaemia has also been suggested to contribute to the development of the disease¹⁸. Processing of proinsulin to insulin in β -cells is catalysed by the sequential actions of prohormone convertases PC1/3 and PC2, which act in concert with carboxypeptidase E. Genetic inactivation of PC2 in mice does not result in a diabetic phenotype¹⁹, suggesting that PC1/3 is more important than PC2 with respect to processing of proinsulin to active insulin. Moreover, a patient displaying a compound heterozygote defect in the PC1/3 gene showed elevated plasma proinsulin levels and perturbed glucose homeostasis²⁰, emphasizing that PC1/3 is critical in proinsulin processing. To investigate a potential insulin-processing defect in the FRID1 mice, we monitored the expression of the proinsulin-processing enzymes PC1/3 and PC2. The expression of PC1/3 (Fig. 2c, d) and to a lesser extent PC2 (data not shown) was reduced in the 10-week-old, overt diabetic FRID1 mice. Expression of PC1/3 and PC2 was also reduced in 5-week-old, normoglycaemic pre-diabetic FRID1 mice, whereas the β -cell expressions of Glut2, PC1/3 and PC2 were normal in the FRID1 mice (data not shown). Together, these results indicate that FGFR1c signalling may be required for normal, high-level expression of PC1/3 and PC2 in β -cells.

To address whether the impaired expression of the processing enzymes might affect insulin processing in the FRID1 mice, we

examined the forms of insulin stored in the β -cells of these mice. We used an antibody directed against intact human proinsulin, which does not crossreact with active insulin¹⁹, and antibodies directed against mouse C-peptide 1 and 2 (ref. 21) to evaluate the forms of insulin present in the β -cells of FRID1 mice (Fig. 2e–j). There was a marked reduction in the apparent amounts of both C-peptide 1 (Fig. 2e, f) and C-peptide 2 (data not shown) in the β -cells of 10-week-old FRID1 mice. In contrast, the β -cells of FRID1 mice were loaded with proinsulin (Fig. 2i) compared with control β -cells (Fig. 2j). Radio immune assay (RIA) analyses of pooled whole pancreatic protein extracts, separated by Bio-gel P30 chromatography, from three separate FRID1 animals with urine glucose levels ranging from 0.1% to 2% revealed a median proinsulin level of 48% for the combined FRID1 mice compared with a wild-type level of 5%. Notably, increased proinsulin immunoreactivity was manifest already at the pre-diabetic 5-week stage (data not shown). These results indicate that downregulation of the processing enzymes PC1/3 and PC2 in FRID1 mice results in impaired processing of proinsulin to its active mature form. This processing defect probably contributes to the development of diabetes in the FRID1 mice. Thus, impaired signalling through FGFR1c in the adult β -cell leads to reduced numbers of β -cells, aberrant glucose sensing and impaired insulin processing, which together lead ultimately to the development of diabetes.

The reduced number of β -cells, the disorganization of the islets and the loss of Glut2, but not glucokinase, expression in the β -cell of the FRID1 mice resembled the phenotype of *Rip1/Ipf1^Δ* mice²². *Rip1/Ipf1^Δ* mice undergo a Cre-mediated β -cell-specific excision of exon 2 of the homeobox gene *Ipf1/Pdx1* and develop diabetes at 10–15 weeks²². We therefore examined whether the expression of FGFR1 and FGFR2 was affected in the β -cells of *Rip1/Ipf1^Δ* mice. The expression of both FGFR1 and FGFR2 was reduced in the β -cells of these mice (Fig. 3a, b; and data not shown). To investigate whether the decrease in FGFR1 expression in *Rip1/Ipf1^Δ* mice leads to perturbed insulin processing, we analysed the expression of prohormone convertases in these mice. The expression of both PC1/3 (Fig. 3c, d) and PC2 (data not shown) was downregulated in *Rip1/Ipf1^Δ* mice. *Rip1/Ipf1^Δ* mice also show a reduced expression of insulin (Fig. 3g, h; and ref. 22), C-peptide 1 (Fig. 3e, f) and C-peptide 2 (data not shown). As in the FRID1 mice, the decrease in prohormone convertase expression was paralleled by an increase in proinsulin immunoreactivity in β -cells of *Rip1/Ipf1^Δ* mice (Fig. 3i, j). RIA analyses performed on pooled insulin extracts derived from diabetic *Rip1/Ipf1^Δ* mice ($n = 3$) revealed that ~65% of the total pancreatic insulin existed in the form of proinsulin. Thus, loss of *Ipf1/Pdx1* and impaired signalling through FGFR1c both lead to diabetes, owing to perturbed glucose sensing and insulin processing in adult β -cells.

We next examined the expression of the FGFR1c high-affinity ligands FGF1, FGF2, FGF4 and FGF5 in FRID1 and *RIP/Ipf1^Δ* mice. All four ligands were expressed at reduced levels as compared with wild-type littermates (Fig. 4a–l). These findings suggest that an autoregulatory FGFR1c signalling feedback loop exists in the β -cell that is required to maintain glucose homeostasis by ensuring proper glucose sensing and insulin processing.

Type-2 diabetes results from insulin resistance and β -cell dysfunction, or a combination of both, which can lead to secondary complications such as retinopathy, nephropathy, neuropathy and cardiovascular disease^{3,4}. Our results provide the first evidence, to our knowledge, that FGFR1, FGFR2 and FGF ligands are expressed in β -cells of the adult pancreas and that attenuation of FGFR1c signalling in the pancreas leads to β -cell defects that are characteristic of type-2 diabetes: a reduced β -cell number, impaired glucose sensing¹² and perturbed proinsulin processing.

Infants with low birth weight have been reported to exhibit relatively fewer β -cells²³, and, in addition, reduced growth in early life has been linked to impaired glucose tolerance²⁴. Together, these

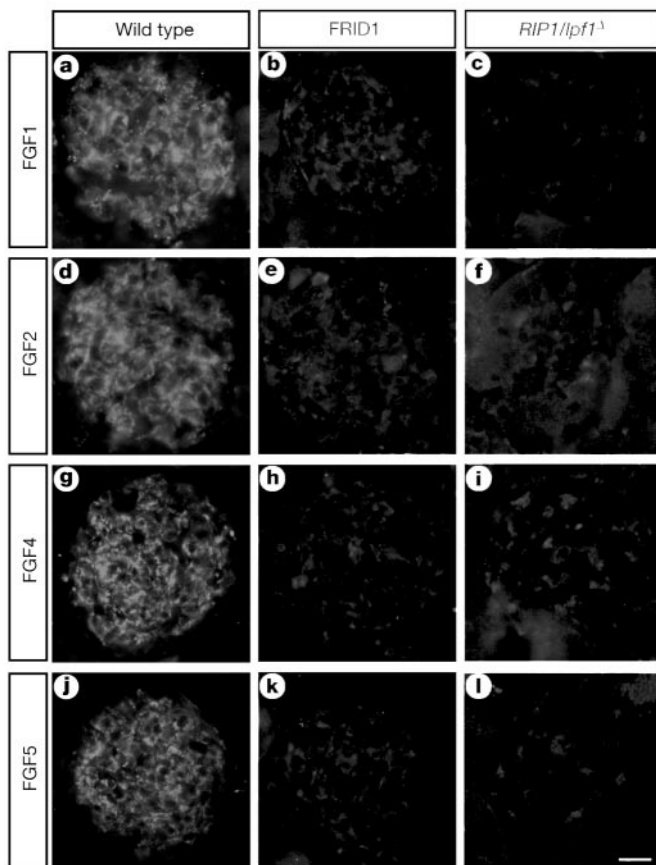


Figure 4 The expression of FGF ligands is perturbed in FRID1 and *RIP/Ipf1^Δ* mice. Attenuation of FGFR1c signalling in β -cells either by expression of dominant-negative FGFR1c as in FRID1 mice (b, e, h, k), or by genetic inactivation of *Ipf1* as in *RIP/Ipf1^Δ* mice (c, f, i, l) leads to reduced expression of FGF1 (a–c), FGF2 (d–f), FGF4 (g–i) and FGF5 (j–l) in the adult β -cell compared with in wild-type mice (a, d, g, j). Images are representative of at least four animals from each group. Scale bars, 20 μ m.

reports point towards a nutritional and/or growth factor stimulus on β -cell expansion and function. Impairment of this stimulation may result in an apparent β -cell dysfunction that predisposes the affected individual to type-2 diabetes. Our results suggest that FGFR1c signalling may be one such key stimulatory factor of β -cell expansion. β -cell hyperplasia has been observed in both humans and rodents under conditions such as pregnancy and non-diabetic obesity when there is an increased demand for insulin^{25,26}. The reduced number of β -cells in 10-week-old, overt diabetic FRID1 mice indicate that FGFR1c signalling may be required for maintaining the competence of β -cells to proliferate in response to hyperglycaemia.

Type-2 diabetic patients exhibit perturbed glucose sensing and also often hyperproinsulinaemia^{3,4,15–18}. Our results provide evidence that FGFR1c signalling in the β -cell is required to ensure normal expression of key components in glucose sensing (Glut2) and insulin-processing machinery (PC1/3 and PC2) and thus to maintain normoglycaemia. In contrast, expression of the dnFGFR2b (FRIND2 mice) under similar conditions does not result in the development of diabetes, thus showing a specificity and selectivity by which FGFR1c signalling ensures glucose homeostasis. In humans, heterozygosity for a non-sense mutation in the *Ipf1* gene, which results in a dominant-negative frameshift, has been linked to maturity-onset diabetes of the young (MODY) 4 (ref. 27), a monogenetic form of diabetes that results from β -cell dysfunction rather than from insulin resistance. Missense mutations in the human *Ipf1* gene have also been implicated in predisposing an individual to type-2 diabetes^{28,29}. We previously showed that *Ipf1/Pdx1* is required for ensuring normal levels of insulin gene expression²². We now provide evidence that *Ipf1/Pdx1* acts upstream of an FGF signalling loop in the β -cell that is required to maintain β -cell function and glucose homeostasis. Whether perturbed FGF signalling contributes to diabetes in humans remains, however, to be determined. □

Methods

Animals

The generation of *Rip1/Ipf1*^Δ mice has been described²², and mutant mice were obtained from our local breeding colony. Mice were genotyped as described²².

Generation of FRID1 and FRIND2 mice

A 4.5-kilobase (kb) *NotI/NaeI* fragment located immediately upstream of the *Ipf1/Pdx1* gene² was subcloned into a vector carrying a poly(A) site and a 1.75-kb *HindIII/EcoRI* fragment of the extracellular FGFR1c variant fused in-frame with the hinge/CH2/CH3 region of the rat IgG1 isoform (FRID1)⁶ or a 1.7-kb *BamHI* (blunt) fragment of the extracellular FGFR2b variant fused in frame with the hinge/CH2/CH3 region of the mouse IgG1 isoform^{7,8} (FRIND2). We generated transgenic mice by pronuclear injection of the purified (*NotI/BamHI*) (1.8 ng ml⁻¹) into F₂ B6/CBA hybrid oocytes as described⁹. The genotype was determined by PCR analysis of genomic DNA extracted from tail biopsies. The primers used were 5'-TAGCGAGGGGAAGAGGAGAT-3' (*Ipf1/Pdx1* primer for 5'), 5'-TTAAGGTCGACACGGTGGTCG-3' (*dnFGFR1c* primer for 3') and 5'-CAAGACCAG GCAGATGAAGCG-3' (*dnFGFR2b* primer for 3').

Immunohistochemistry

Immunohistochemical localization of antigens, double-label immunohistochemistry and confocal microscopy were carried out essentially as described²². The primary antibodies used were rabbit anti-IPF1, rabbit anti-ISL1, rabbit anti-Nkx6.1 (all from ref. 22); rabbit anti-glucagon, guinea-pig anti-glucagon, guinea-pig anti-insulin (all from Linco); rabbit anti-PC2, rabbit anti-PC1/3 (both from ref. 30); rabbit anti-FGFR1, rabbit anti-FGFR2, goat anti-FGF1, goat anti-FGF2, goat anti-FGF4, goat anti-FGF5, goat anti-FGF7, goat anti-FGF10 (all from Research Diagnostics); rabbit anti-Glut2, sheep anti-glucokinase (both from ref. 22); rabbit anti-amylase (Sigma); rabbit anti-C-peptide 1, rabbit anti-C-peptide 2 (both ref. 21); and mouse anti-proinsulin¹⁹. The secondary antibodies used were fluorescein anti-guinea-pig, fluorescein anti-rat, fluorescein anti-mouse, Cy3 anti-rabbit, Cy3 anti-mouse, biontinylated anti-goat and streptavidin CY3 (all from Jackson).

Glucose measurements

Urine glucose was routinely measured on non-fasted wild-type and transgenic animals using Diastix (Bayer) > 2% (or > 11.1 mM). Blood samples were obtained from the tail vein of both overnight-fasted (wild-type and transgenic animals) and non-fasted animals and glucose levels were measured using a Precision Plus glucometer (Medisense).

Insulin measurements and chromatography

Pancreatic insulin²² was prepared, and Bio-gel P30 chromatography¹⁹ was performed essentially as described^{19,22}.

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Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase

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Disulphide bonds in secreted proteins are considered to be inert because of the oxidizing nature of the extracellular milieu. An exception to this rule is a reductase secreted by tumour cells that reduces disulphide bonds in the serine proteinase plasmin^{1,2}. Reduction of plasmin initiates proteolytic cleavage in the kringle

5 domain and release of the tumour blood vessel inhibitor angiostatin³. New blood vessel formation or angiogenesis is critical for tumour expansion and metastasis^{4,5}. Here we show that the plasmin reductase isolated from conditioned medium of fibrosarcoma cells is the glycolytic enzyme phosphoglycerate kinase⁶. Recombinant phosphoglycerate kinase had the same specific activity as the fibrosarcoma-derived protein. Plasma of mice bearing fibrosarcoma tumours contained several-fold more phosphoglycerate kinase, as compared with mice without tumours. Administration of phosphoglycerate kinase to tumour-bearing mice caused an increase in plasma levels of angiostatin, and a decrease in tumour vascularity and rate of tumour growth. Our findings indicate that phosphoglycerate kinase not only functions in glycolysis but is secreted by tumour cells and participates in the angiogenic process as a disulphide reductase.

Plasmin contains five consecutive kringle domains followed by a serine proteinase module and is processed in the conditioned medium of tumour cells producing angiostatin fragments consisting of kringle domains 1–4 and parts of kringle 5 (refs 1, 2, 7). Plasmin proteolysis occurs in two stages. First, the Cys 461/Cys 540 and Cys 511/Cys 535 disulphide bonds in kringle 5 of plasmin are reduced by a plasmin reductase². Second, reduction of the kringle 5 disulphide bonds trigger cleavage at Arg 529/Lys 530 in kringle 5, and also at two other positions carboxy-terminal to Cys 461, by a

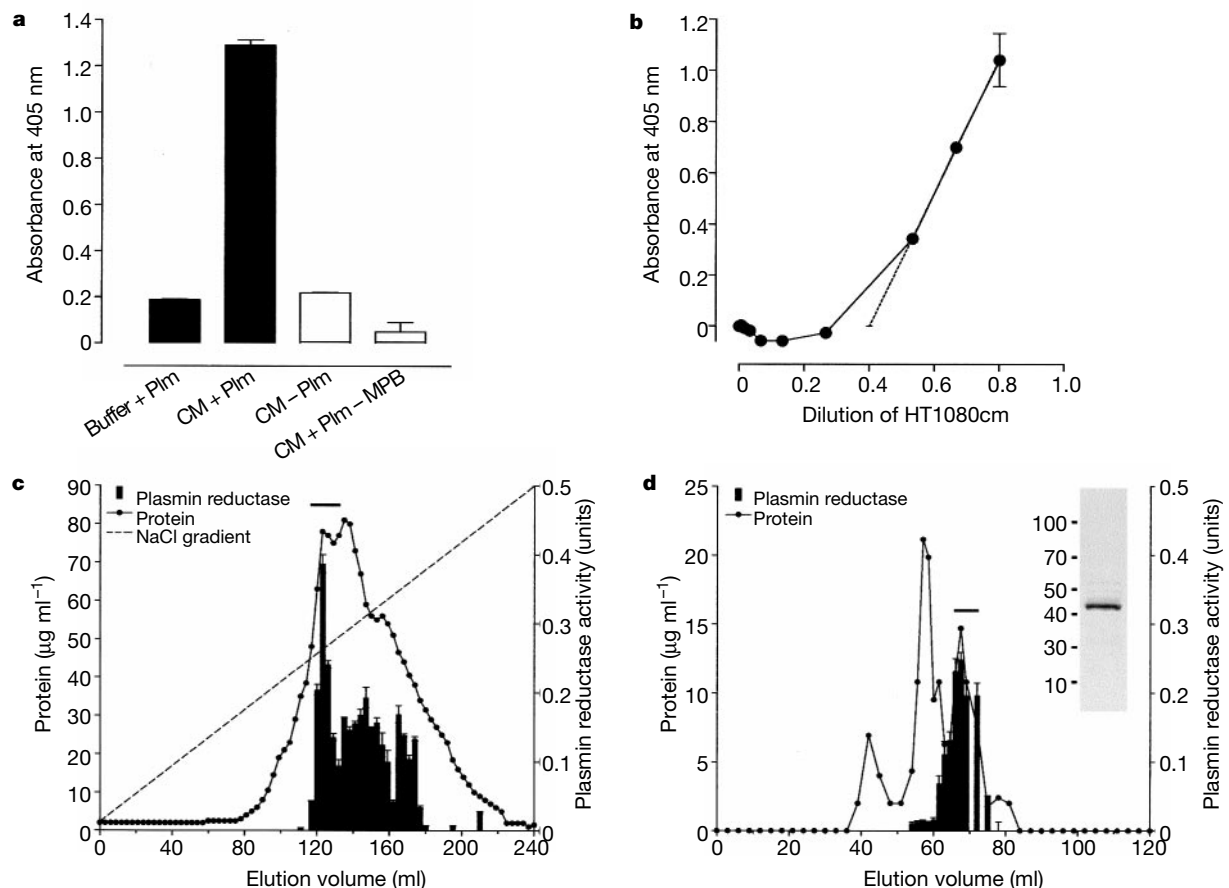


Figure 1 Assay and purification of plasmin reductase. **a**, HT1080 conditioned medium (HT1080cm) was incubated with plasmin (Plm; $2 \mu\text{g ml}^{-1}$) in HEPES-buffered saline for 30 min at 37°C . The control reaction was plasmin incubated in HEPES-buffered saline. The angiostatin fragments formed were labelled with MPB and detected by enzyme-linked immunosorbent assay (ELISA). For controls, HT1080cm was incubated without plasmin, and the MPB labelling of a HT1080cm/plasmin reaction was omitted on one occasion (open bars). Results are the mean $\pm$ s.d. of triplicate determinations. **b**, Plasmin ($2 \mu\text{g ml}^{-1}$) was incubated in dilutions of HT1080cm in HEPES-buffered saline for 30 min at 37°C , and the angiostatin fragments were labelled with MPB and detected by ELISA.

Data are corrected for background absorbance (incubation of plasmin in HEPES buffer alone; see **a**). Dotted line represents the linear least squares fit to the three right-hand data points ($r^2 = 0.99$). Results are the mean $\pm$ s.d. of triplicate determinations. **c**, Conditioned medium from HT1080 cells was applied to Cibachron Blue-Sepharose and the bound proteins were eluted with a linear NaCl gradient. Horizontal bar indicates fractions that were pooled. **d**, The DTT eluate from the activated thiopropyl-Sepharose column was gel filtered on Sephacryl S-200 HR. Horizontal bar indicates fractions that were pooled. Inset, pooled plasmin reductase resolved on 4–15% SDS-PAGE (BioRad) and silver stained (Pierce).

serine proteinase^{2,7}. Autoproteolysis can account for the cleavage, although other serine proteinases may be involved^{2,7}. The kringle 1–4₂ fragment ending at Arg 529 inhibits bovine capillary endothelial cell proliferation *in vitro* and angiogenesis *in vivo*⁸.

Plasmin reductase activity in the conditioned medium of tumour cells was measured by formation of free thiols in angiostatin using the avidin–biotin interaction in a microtitre plate format. Briefly, plasmin was incubated with samples containing plasmin reductase and the resulting angiostatin fragments were labelled with the thiol-specific biotin-linked maleimide, 3-(*N*-maleimidylpropionyl)biotin (MPB)^{1,2}. The MPB-labelled angiostatin fragments were bound to an anti-angiostatin monoclonal antibody coated on microtitre plate wells, and the incorporated MPB was measured using streptavidin–peroxidase (Fig. 1a). There was a linear relationship ($r^2 = 0.99$) between plasmin reductase concentration and absorbance value after a threshold concentration of plasmin reductase was achieved (Fig. 1b). Plasmin reductase activity was measured in this linear range.

We observed that plasmin reductase activity in HT1080 fibrosarcoma conditioned medium was inhibited by the adenine nucleotides NAD(H) or ATP, and inactivated by the thiol-modifying reagents iodoacetamide or *N*-ethylmaleimide (data not shown). These results suggested that plasmin reductase would have affinity for the chromatography matrices Cibachron Blue-Sepharose and activated thiopropyl-Sepharose. We concentrated 20 l of conditioned medium from HT1080 cells (840 mg) and applied it to Cibachron Blue-Sepharose. The bound proteins were eluted with a linear NaCl gradient. Plasmin reductase activity eluted as a broad peak starting at ~1 M NaCl (Fig. 1c). The leading edge of the peak of plasmin reductase activity was pooled (6 mg with 120-fold increase in specific activity) and batch adsorbed to activated thiopropyl-Sepharose. The matrix was developed with buffers of increasing reducing potential to elute the covalently bound proteins. Most of the plasmin reductase activity eluted with 20 mM dithiothreitol (DTT; 2 mg with a 710-fold increase in specific activity). The eluate was concentrated and gel filtered on Sephacryl S-200 HR (Fig. 1d). The plasmin reductase activity was well resolved and associated with a single polypeptide chain with a relative molecular mass of about

42,000 ($M_r \approx 42K$) by SDS–polyacrylamide gel electrophoresis (Fig. 1d, inset). About 20 μ g of plasmin reductase was isolated with a 5,000-fold increase in specific activity.

The intact 42K protein was refractive to amino-terminal sequencing. The protein was digested with CNBr, the fragments resolved by reverse-phase high performance liquid chromatography and 34 N-terminal residues of 4 internal peptides were determined. Plasmin reductase was found to have 100% sequence identity to phosphoglycerate kinase⁶ (PGK; ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (data not shown). We cloned human PGK complementary DNA by polymerase chain reaction with reverse transcription (RT–PCR) from HT1080 RNA. The hPGK cDNA open reading frame, which codes for 418 amino acids, was subcloned into the plasmid vector, pET11a, which was

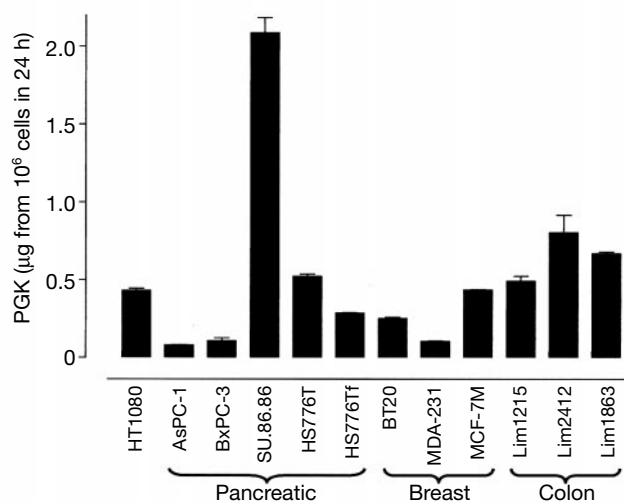


Figure 3 PGK is secreted by cultured tumour cells. Serum-free conditioned medium from HT1080 cells and several human pancreatic, breast and colon tumour cells was collected for 24 h, and the mass of PGK secreted from 10⁶ cells was determined²¹. Results are the mean $\pm$ s.d. of triplicate determinations.

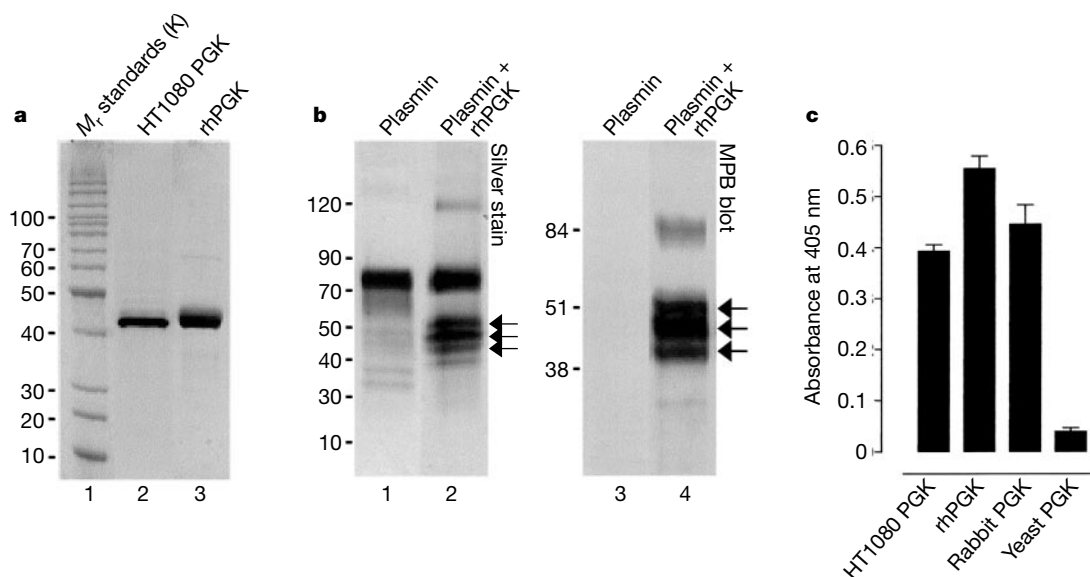


Figure 2 Recombinant human PGK has plasmin reductase activity. Human PGK cDNA was cloned by RT–PCR from HT1080 RNA. Human recombinant PGK protein was produced in *E. coli* and purified. **a**, HT1080 PGK and rhPGK (2 μ g) were resolved on 4–15% SDS–PAGE and Coomassie stained. **b**, Plasmin (20 μ g) was incubated with rhPGK (40 μ g) in 0.5 ml of HEPES-buffered saline for 30 min at 37 °C, and the angiostatin fragments were labelled with MPB (see Fig. 1a). Plasmin products were collected on

lysine–Sepharose beads² and either separated on 4–15% SDS–PAGE and silver stained (left), or separated on 10% SDS–PAGE and blotted with streptavidin–peroxidase to detect MPB² (right). Arrows indicate the three angiostatin fragments^{1,2}. **c**, Plasmin (2 μ g ml^{–1}) was incubated with HT1080 PGK, rhPGK, rabbit PGK or yeast PGK (5 μ g ml^{–1}) in HEPES-buffered saline for 30 min at 37 °C, and the angiostatin fragments were labelled with MPB and detected by ELISA. Results are the mean $\pm$ s.d. of triplicate determinations.

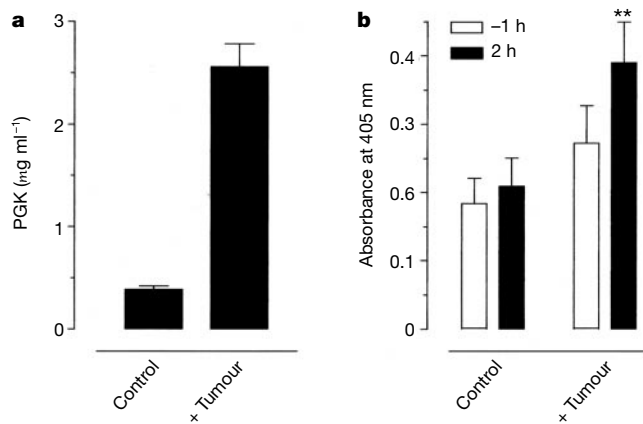


Figure 4 PGK is secreted by tumours, and administration of PGK to tumour-bearing mice causes an increase in plasma levels of angiostatin that contains free thiols. HT1080 tumours were established subcutaneously in the proximal midline dorsum of SCID mice²². Blood was collected from the retro-orbital plexus of mice without ($n = 4$) and with ($n = 4$) tumours; 1 h later the mice were administered 10 mg per kg of rhPGK in PBS through a 0.2-ml intraperitoneal injection. The mice were bled again after 2 h. Blood was collected into PBS containing EDTA (20 mM), aprotinin (5 μ M) and MPB (200 μ M), and cysteine (400 μ M) was added after 60 min to quench unreacted MPB. Plasma was separated and assayed for PGK (**a**) or MPB-labelled angiostatin (**b**). Mean tumour mass, 5.7 ± 1.0 g; mean mass of control and tumour-bearing mice, 23 ± 3 g and 22 ± 2 g, respectively. Results are the mean $\pm$ s.e.m. of the four mice plasmas. Double asterisks; $P < 0.05$.

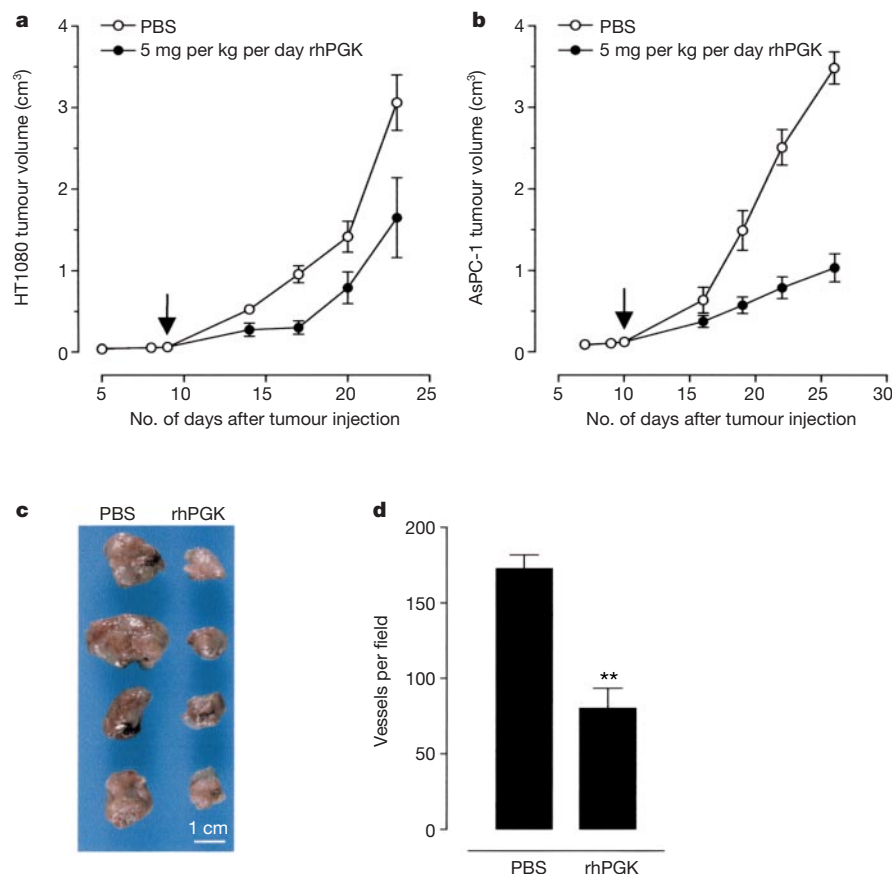


Figure 5 Administration of PGK to tumour-bearing mice results in decrease in tumour vascularity and rate of tumour growth. HT1080 (**a**) or AsPC-1 (**b**) tumours were established in the proximal midline dorsum of SCID mice²². When the tumours were ~ 0.1 cm³ in volume, mice were randomized into two groups ($n = 4$) and treated with either vehicle (PBS) or 5 mg per kg per day of rhPGK through 0.2-ml intraperitoneal

then transfected into the *Escherichia coli* strain BL21 (DE3). Recombinant protein was expressed, extracted and purified. Recombinant human PGK (rhPGK) resolved at the same size as the HT1080 protein on SDS-PAGE (Fig. 2a) and had the same plasmin reductase activity as the HT1080 protein (Fig. 2b, c). These findings show that plasmin reductase is PGK. Rabbit muscle PGK had comparable specific plasmin reductase activity to rhPGK or HT1080 PGK; yeast PGK had negligible activity (Fig. 2c). Human and rat PGK share 85% sequence identity, whereas the human and yeast enzyme share 65% sequence identity.

Reductase active sites are characterized by closely spaced cysteines in the consensus sequence, Cys-Gly-X-Cys⁹. The cysteine thiols cycle between the reduced dithiol and oxidized disulphide bond, in coordination with a dithiol or disulphide of a protein substrate; this can result in the reduction, formation or interchange of disulphide bonds in the protein substrate. There are no cysteines of this type in PGK, which implies that the mechanism by which PGK reduces disulphide bonds in plasmin is distinct from that of conventional reductases. As the plasmin reductase activity of PGK is inactivated by thiol-modifying agents, however, one or more of the PGK cysteine residues may have a role in plasmin reduction.

Rates of secretion of PGK by tumour cells varied considerably (Fig. 3). Cultured HT1080 cells secreted 0.43 ± 0.01 μ g of PGK per 10^6 cells in 24 h. We compared this rate of secretion with rates of secretion of PGK from four human pancreatic (AsPC-1, BxPC-3, SU.86.86 and Hs766T), three human breast (BT20, MDA-231 and MCF-7M) and three human colon (Lim1215, Lim2412 and Lim1863) tumour cell lines. The secretion rates

injections. Arrow indicates the start of treatment. Results represent the mean $\pm$ s.e.m. of the tumour volumes. **c**, Mice in **b** were killed at day 16 of treatment and the AsPC-1 tumours were excised. **d**, Histological sections of the AsPC-1 tumours in **c** were analysed for vascularity by immunostaining for CD31²³. Double asterisks; $P < 0.05$.

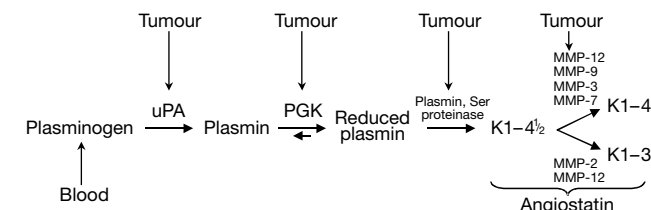
varied 26-fold among the cell lines. SU.86.86 cells secreted the most PGK, $2.09 \pm 0.10 \mu\text{g}$ per 10^6 cells in 24 h, while AsPC-1 cells secreted the least, $0.08 \pm 0.01 \mu\text{g}$ per 10^6 cells in 24 h. In addition, a cell line cloned from Hs766T, Hs766Tf, secreted 82% more PGK in 24 h than did the parent line. There was no discernible decrease in cell viability or significant release of lactate dehydrogenase from the cells after 24 h of culture. These observations implied that secretion of PGK was a specific property of living cells and was not due to leakage from dead cells. Notably, the rate of secretion of PGK by the HT1080 (ref. 2), breast² and colon tumour (data not shown) cells correlated with the plasmin reductase activity secreted by these cells. Moreover, the rate of secretion of PGK by the pancreatic tumour cells was inversely correlated with the tumorigenicity of the cells grown subcutaneously in the proximal midline dorsum of severe combined immunodeficient (SCID) mice. The tumorigenicity was in the order AsPC-1 > BxPC-3 >> SU.86.86 (data not shown).

The plasma of mice bearing HT1080 tumours contained 6.6-fold more PGK than did mice without tumours (Fig. 4a). This finding implied that relatively small amounts of PGK was secreted from normal tissues, which is in accordance with the observation that transformed cells secrete much more PGK (Fig. 3) than do primary cells². To determine whether PGK could reduce plasmin *in vivo*, mice with and without HT1080 tumours were injected with rhPGK. We thought that the rhPGK would facilitate reduction of plasmin produced by the HT1080 tumours and result in increased plasma levels of angiostatin containing free thiols. The plasma levels of MPB-labelled angiostatin were 48% higher on average in tumour-bearing mice as compared with mice without tumours (Fig. 4b). Administration of rhPGK to tumour-bearing mice resulted in an 86% increase in the plasma levels of MPB-labelled angiostatin after 2 h ($P < 0.05$). In contrast, administration of rhPGK to mice without tumours had no significant effect on plasma levels of MPB-labelled angiostatin. These findings support the proposed role for PGK in angiostatin formation and imply that blood levels of angiostatin can be increased by systemic administration of PGK. This led us to test whether treatment of tumour-bearing mice with PGK would inhibit tumour angiogenesis and growth.

The growth of human fibrosarcoma (HT1080) and pancreatic carcinoma (AsPC-1) tumours in immunocompromised mice was suppressed by systemic administration of rhPGK. Intraperitoneal administration of 5 mg rhPGK per kg per day caused ~50% inhibition of the rate of HT1080 tumour growth (Fig. 5a) and ~70% inhibition of the rate of AsPC-1 tumour growth (Fig. 5b). There were no apparent adverse side-effects of administration of rhPGK to the mice, and no macroscopic (Fig. 5e) or microscopic (data not shown) signs of necrosis of the rhPGK-treated tumours. Immunohistochemical staining of the AsPC-1 tumours (Fig. 5c) for vascularity indicated a marked reduction in angiogenesis in the rhPGK-treated tumours (Fig. 5d). There were 80 ± 13 vessels per field in the rhPGK-treated tumours compared with 173 ± 9 vessels per field in the vehicle-treated tumours ($P < 0.05$). It is interesting that AsPC-1 tumorigenesis was more susceptible to suppression by systemic administration of PGK than was HT1080 tumorigenesis. This may reflect the fivefold reduced secretion of PGK by AsPC-1 cells as compared with HT1080 cells (Fig. 3).

PGK has traditionally been studied as the sixth enzyme of the glycolytic pathway in which it equilibrates phosphate transfer between position 1 of 1,3-bisphosphoglycerate and the γ -phosphate of MgATP^{2-} . PGK has also been shown to influence DNA replication and repair in mammalian cell nuclei^{10,11}, and stimulate viral messenger RNA synthesis in the cytosol¹², and has been detected on the surface of *Candida albicans* where it extends through the cell wall¹³. The hypoxic nature of solid tumours triggers expression of vascular endothelial cell growth factor, which stimulates angiogenesis, and glycolytic enzymes including PGK, which facilitate anaerobic production of ATP^{14} . Conversely, hypoxia markedly inhibited secretion of PGK from HT1080 cells and angiostatin production in

the conditioned medium (data not shown). This result indicated that secretion of PGK is regulated independently and inversely of its production and is consistent with the correlation between tumour hypoxia and angiogenesis. These and other findings support the following model for angiostatin formation by tumours.



We propose a sequential order of events beginning with conversion of plasminogen to plasmin^{1,7,15}, followed by reduction of plasmin by PGK, then serine proteinase-dependent release of kringle 1–4^{1/2} (refs 1, 2, 7, 15), and finally matrix metalloproteinase-dependent trimming of kringle 1–4^{1/2} to either kringle 1–4 or 1–3 (refs 16–18). □

Methods

Assay for plasmin reductase activity

Angiostatin fragments generated from plasmin were labelled with MPB^{1,2}, immobilized on wells coated with a murine anti-angiostatin monoclonal antibody and detected using StreptABComplex/HRP (Dako)². The murine monoclonal antibody was generated against human angiostatin² as described¹⁹. The antibody, designated 8.19, binds with equivalent affinity to soluble angiostatin and plasmin and with ~10-fold lower affinity to plasminogen.

Purification of PGK from HT1080 conditioned medium

Conditioned medium of HT1080 cells was prepared using Nunc Cell Factories. HT1080 cells (~100,000 cells per cm² of cell factory area) were seeded into cell factories in DMEM with 10% fetal calf serum. Cells at ~80% confluence were incubated with Hank's balanced salt solution at 37°C, 5% CO₂ for 30 h. The conditioned medium was collected, centrifuged and passed through a 0.2- μm filter to remove detached cells and cellular debris. Twenty litres of conditioned medium was concentrated to 350 ml using an Amicon spiral-wound concentrator with a 10K cutoff membrane. The proteinase inhibitors, leupeptin (10 μM), phenylmethylsulphonyl fluoride (1 mM), EDTA (2 mM) and soybean trypsin inhibitor (10 $\mu\text{g ml}^{-1}$) were added to the concentrated medium to minimize proteolytic degradation of the plasmin reductase. The concentrated medium was dialysed against 20 mM HEPES, 0.05 M NaCl, 1 mM EDTA, 0.02% Na₂S₂O₅, pH 7.4 buffer and applied to an 80-ml column of Cibachron Blue-Sepharose (2.5 × 17 cm) (Pharmacia) equilibrated with the same buffer. The column was washed with three bed volumes of the HEPES buffer at a flow rate of 0.5 ml min⁻¹ to elute unbound proteins, and developed with a 240-ml linear NaCl gradient from 0.05 M to 2 M in the HEPES buffer. The fractions containing plasmin reductase activity (~17 ml) were dialysed against 20 mM HEPES, 0.14 M NaCl, 1 mM EDTA, pH 7.4 buffer and batch adsorbed for 60 min at 20°C to 6 ml of swollen thiopropyl-Sepharose (Pharmacia) equilibrated with the same buffer. The matrix was packed into a column and washed with 10 bed volumes of the equilibration buffer at a flow rate of 0.1 ml min⁻¹. Proteins were eluted with buffers of increasing reducing potential. The buffers were 20 mM HEPES, 0.14 M NaCl, 1 mM EDTA, pH 7.4 containing 5 mM cysteine, 50 mM reduced glutathione or 20 mM DTT. One void volume (2 ml) of buffer containing reducing agent was run into the column and allowed to react with the matrix for 30 min at 20°C before eluting the protein with 10 ml of buffer without reducing agent. Plasmin reductase activity was eluted with DTT. The eluate was concentrated to 2.4 ml, dialysed against 20 mM HEPES, 0.14 M NaCl, 1 mM EDTA, 0.02% Na₂S₂O₅, pH 7.4 buffer and applied to a 120-ml-column of Sephacryl S-200 HR (1.5 × 70 cm) at a flow rate of 0.2 ml min⁻¹.

Cloning and expression of recombinant human PGK

A 1.33-kilobase (kb) hPGK cDNA was isolated by RT-PCR from total RNA extracted from HT1080 cells². The forward and reverse primers were 5'-AGTACATATGTCGCTTTCT AACAGCTG-3' (positions 80–100) and 5'-AGTAGGATCCCTAATGCCAAGTGGAG ATGCA-3' (positions 1,409–1,389), respectively. The hPGK cDNA contained the open reading frame of hPGK. Integrity of the cDNA was confirmed by automatic sequencing (ABI-377 Automatic Sequencer; Applied Biosystems) and was the same as the reported sequence⁶. The hPGK cDNA was subcloned into the plasmid vector, pET11a, which was then transfected into *E. coli* strain, BL21 (DE3) (Novagen). Recombinant protein was produced²⁰ and purified as for the HT1080 protein. The rhPGK was depleted of endotoxin with Detoxi-Gel (Pierce).

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Rapid exchange of histone H1.1 on chromatin in living human cells

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The considerable length of DNA in eukaryotic genomes requires packaging into chromatin to fit inside the small dimensions of the cell nucleus. Histone H1 functions in the compaction of chromatin into higher order structures derived from the repeating 'beads on a string' nucleosome polymer. Modulation of H1 binding

activity is thought to be an important step in the potentiation/depotentiation of chromatin structure for transcription^{1–4}. It is generally accepted that H1 binds less tightly than other histones to DNA in chromatin and can readily exchange in living cells^{5–8}. Fusion proteins of Histone H1 and green fluorescent protein (GFP) have been shown⁹ to associate with chromatin in an apparently identical fashion to native histone H1. This provides a means by which to study histone H1–chromatin interactions in living cells. Here we have used human cells with a stably integrated H1.1–GFP fusion protein to monitor histone H1 movement directly by fluorescence recovery after photobleaching in living cells. We find that exchange is rapid in both condensed and decondensed chromatin, occurs throughout the cell cycle, and does not require fibre–fibre interactions. Treatment with drugs that alter protein phosphorylation significantly reduces exchange rates. Our results show that histone H1 exchange *in vivo* is rapid, occurs through a soluble intermediate, and is modulated by the phosphorylation of a protein or proteins as yet to be determined.

To monitor H1 *in vivo*, we constructed a chimaeric protein of the major human histone subtype, H1.1, with GFP fused at the carboxy terminus of the histone tail. We then generated stable transformants in SK-N-SH human neuroblastoma cells as described¹⁰. The H1.1–GFP content was variable between cells, but did not exceed 10% of the total H1 content in any of the cells examined. Fluorescence microscopy shows that H1.1–GFP binds chromatin throughout the cell cycle in a manner similar to that of normal histone H1. This is shown in Fig. 1 by the fluorescence observed on chromosomes as cells traverse mitosis and into the next G1. Notably, both the interphase and mitotic cells show essentially complete association of H1.1–GFP with chromatin. This was also observed with Raji and 8226 lymphocyte cell lines stably expressing the same H1.1–GFP construct (data not shown). The small amount of extrachromosomal fluorescence (for example, see the telophase cell) is due to an autofluorescent component of these cells that emits at a slightly shorter wavelength than GFP, as determined by visual inspection, and is therefore not derived from H1.1–GFP.

We further demonstrated the proper assembly of histone H1.1–GFP into chromosomes by measuring its salt-elution properties. Micrococcal nuclease-generated soluble chromatin fragments were bound to hydroxylapatite in the presence of 0.1 M phosphate and eluted with increasing concentrations of NaCl in the presence of 0.1 M phosphate¹¹. Under these conditions, the association of chromatin with the matrix is maintained by the DNA, while NaCl dissociates the histones from the DNA. Typically, the H1 histones elute from chromatin between 0.3 and 0.45 M NaCl, the H2A–H2B dimers elute at roughly 0.8 M NaCl, and the H3–H4 tetramers elute at around 1.2 M NaCl (ref. 11). When chromatin was isolated from cells that stably expressed histone H1.1–GFP, and then bound to hydroxylapatite and washed with increasing concentrations of NaCl, H1.1–GFP eluted between 0.3 and 0.4 M NaCl (Fig. 1, inset). Similar to H1.1–GFP, the peak elution fraction for the native H1 histones was 0.35 M NaCl (data not shown), indicating that the GFP tag does not affect the binding affinity of the H1 variant.

Early *in vitro* studies using isolated chromatin showed that H1 histones assemble the chromatin into higher order structures, and that they may be involved in regulating transcription. To understand better how these proteins function, we performed experiments that directly monitor the dynamic properties of H1 mobility in living cells. For quantitative analysis of histone H1.1 exchange, we carried out fluorescence recovery after photobleaching (FRAP) experiments (see Methods). FRAP experiments can be used to define the average mobility of molecules in living cells. Using this approach, it has been shown that certain non-histone proteins in the nucleus can move rapidly with free diffusion. For example, GFP recovers in less than 1 s under the conditions used here¹⁰. Other

proteins, such as high mobility group-17 and alternate splicing factor, move considerably slower than would be expected for their molecular masses^{10,12}, indicating that they undergo reversible binding to less mobile components of the cell nucleus. As shown by FRAP, movement of histone H1.1–GFP occurs from the unbleached to the bleached region (Fig. 2a). There is a corresponding decrease in fluorescence of the unbleached area as the bleached region increased in intensity, showing that recovery of fluorescence occurred from movement of H1 molecules from the unbleached into the bleached region. The histone H1.1–GFP redistributed evenly across the cell nucleus in roughly 10 min. This is in marked contrast to histone H2B, which showed little or no recovery over a 30-min period (Fig. 2b). In control experiments in which whole nuclei were bleached, no recovery of fluorescence was seen (data not shown). This eliminates the possibility that *de novo* synthesis of H1.1–GFP or refolding of GFP accounts for any of the fluorescence recovered in the duration of the previous experiments.

It is generally held that photobleaching does not affect the structure of GFP chimaeric proteins. In these experiments, it is important to remember that it is not the photobleached H1.1 that is being monitored. Rather, it is the movement of the native fluorescent H1.1–GFP into regions that have been photobleached. However, we cannot completely exclude the possibility that photobleaching alters H1.1–GFP structure and binding, and thus may influence the binding properties of the unbleached H1.1–GFP molecules.

Histone H1 exchange may occur through direct physical interactions between chromatin fibres as suggested by *in vitro* analysis¹³, or it may occur by exchanging with a soluble pool of H1. Experiments on histone H1 mobility to date confirm the presence of a population of H1 that is capable of transferring from one chromatin binding site to another ('mobile fraction'). However, it is not known whether this DNA-binding protein dissociates from chromatin and travels through the nucleoplasm before resuming binding, or whether it requires fibre–fibre interactions to 'jump' to the next binding site. The two possibilities can be conclusively discriminated by examining lagging chromosomes during mitosis (Fig. 3a, b) and isolated apoptotic bodies during apoptosis (Fig. 3c). We performed FRAP experiments on spatially isolated chromatin from mitotic and apoptotic cells. Complete recovery of the H1.1–GFP was observed in both cases with kinetics similar to those of H1 movement in larger chromatin masses. Photobleaching of these isolated chromatin bodies clearly demonstrate that recovery occurs despite isolation from unbleached chromatin during the course of the experiment. These results show that fibre–fibre interactions are not a strict requirement for the histone H1 exchange process *in vivo*, and indicate that histone H1 exchange occurs through a pathway that involves dissociation, diffusion through the nucleoplasm, and then reassociation with chromatin.

The rapid mobility of histone H1 in the nucleus did not appear to be an energy-dependent process, as depletion of cellular pools of ATP (15–60 min carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

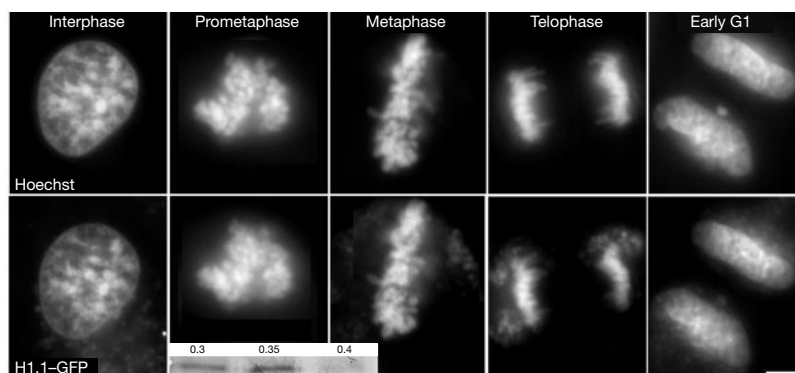


Figure 1 Fluorescence microscopy of subcellular distribution of H1.1–GFP throughout the cell cycle. SK-N-SH human neuroblastoma cells stably expressing histone H1.1–GFP were counterstained for DNA by adding Hoechst 33342 dye to the culture medium. Digital fluorescence microscopy of expressing cells at different stages of the cell cycle are

shown. Inset shows the hydroxylapatite elution profile of H1.1–GFP resolved on a 10% polyacrylamide SDS gel. The concentration of NaCl in the elution buffer is given above each lane. The identity of the band as H1.1–GFP was confirmed by immunoblotting with a total H1 antibody. Scale bar, 10 μ m.

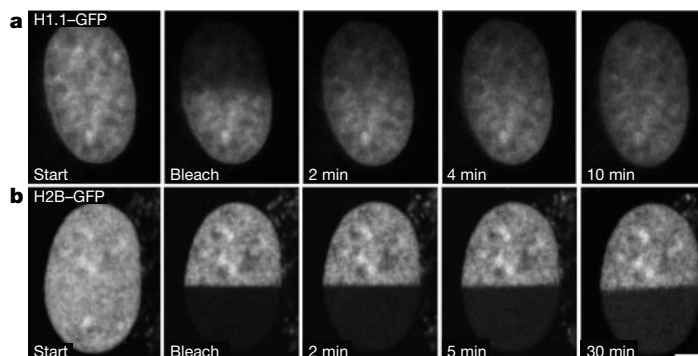


Figure 2 Histone H1.1 exchange *in vivo*. **a**, An SK-N-SH cell expressing histone H1.1–GFP photobleached over a region covering roughly one-half of the nucleus. Movement of fluorescent H1.1–GFP was monitored until evenly distributed across the entire cell. Images were taken immediately before photobleaching, immediately after photobleaching, and at indicated intervals during recovery. **b**, An SK-N-SH cell stably expressing

histone H2B–CFP photobleached over a region covering roughly one-half of the nucleus. Recovery of fluorescence was monitored for 30 min. Images were taken immediately before photobleaching, immediately after photobleaching, and at indicated intervals during recovery. Scale bar, 5 μ m.

(FCCP) + 2-deoxyglucose (2DG)) did not significantly affect the rate of recovery of fluorescence after photobleaching (data not shown). However, a reduced rate of exchange was seen with prolonged incubation (4 h) with ATP-depleting agents (Fig. 4, Table 1). Immunofluorescence examination with antibodies¹⁴ to phosphorylated histone H1 showed a reduction in staining in these cells after prolonged incubation with ATP-depleting agents but not after brief treatment (data not shown). In addition, prolonged treatment significantly reduced the mobile fraction of H1 present in chromatin, which further suggested that phosphorylation influences H1 binding (Table 1). A failure to reach the predicted 100% recovery is assumed to reflect the presence of a population of photobleached molecules that cannot be displaced from the photobleached region (that is, immobile population). It is therefore possible that post-translational modification of histone H1 or a protein that modulates H1 binding may be involved in regulating the movement of histone H1.

To test this hypothesis, we used two separate kinase inhibitors, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) and staurosporine, both of which inhibit histone H1 phosphorylation^{15,16}. FRAP experiments of treated cells showed an approximate doubling in the time required for 50% of the histone H1.1-GFP molecules to exchange (Fig. 4, Table 1). The differences in the mean times required for 50% recovery of H1 fluorescence were statistically significant when comparing drug-treated with untreated cells (Table 1). These results indicate that protein phosphorylation may promote the displacement of histone H1.1 from chromatin. Phosphorylation of the C-terminal tail disrupts the interactions between this highly basic domain and DNA to alter chromatin

organization¹⁷, and may have similar effects as the binding of chromatin with just the globular H1 domain alone. *In vitro* studies with reconstituted systems showed that the globular domain of H1 alone is sufficient to bind chromatin¹⁸. Notably, deletion of the highly positively charged C terminus of H1.1, which is the principal site of mitotic phosphorylation, markedly increases the mobility of histone H1 (Table 1) and results in a population of H1-GFP that is not bound to chromosomes during mitosis (data not shown).

One observation superficially suggests that H1 is not the target of the phosphorylation that modulates H1 binding. In early G1, H1 is minimally phosphorylated at steady state. Despite this, recovery rates were similar in early G1 as compared with interphase nuclei (Table 1). It is possible that there is sufficient H1 phosphorylation to displace the H1 molecules, but that high rates of dephosphorylation result in a very short half-life for the phosphorylated state and consequently low levels of steady-state phosphorylation. Indeed, incubating interphase cells with phosphatase inhibitors can increase the phosphorylation of histones^{19–22}, suggesting that phosphatases are constitutively active to regulate the overall level of histone phosphorylation.

We have shown that H1 is dynamically associated with chromatin in living cells and that the duration of H1 binding *in vivo* is modulated by protein phosphorylation. The absence of a strict

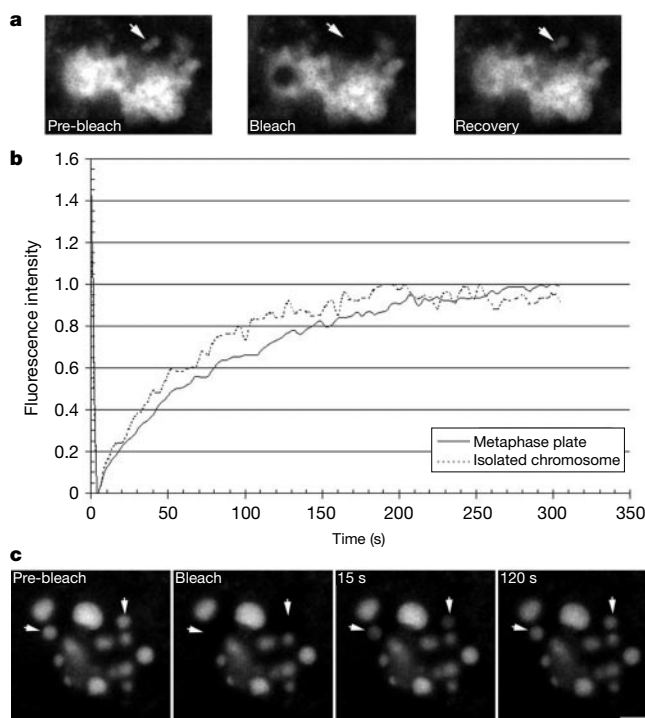


Figure 3 Histone H1.1 exchange occurs between physically separated regions of chromatin. **a**, A spot photobleaching experiment on a metaphase cell containing a lagging chromosome is shown. Two spots were photobleached, one in the chromosome mass and the other containing the lagging chromosome (arrow). Images were taken immediately before and after photobleaching, and at the end of the recovery period. **b**, Relative intensity plot of the photobleaching experiment in **a**. **c**, A spot photobleaching experiment on a spontaneously arising apoptotic cell. Two spots containing individual apoptotic chromatin bodies (arrows) were photobleached and monitored for fluorescence recovery. Scale bar, 5 μ m.

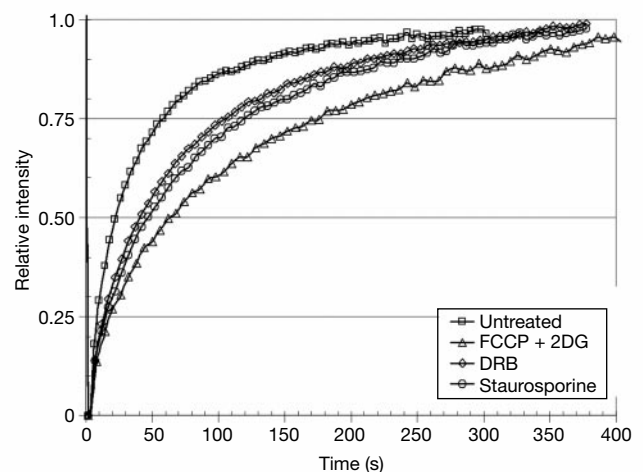


Figure 4 Recovery profiles of spot bleaching experiments under untreated and drug-treated conditions. Two 2- μ m spots were photobleached simultaneously and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity, reached at the end of recovery, represents 1.0. Thus, the initial values were greater than 1.0 and are not shown on the graph to illustrate better differences in the recovery curves. To generate the curves shown, two spots were simultaneously photobleached in each cell nucleus. The number of nuclei used to generate each plot was 34 for the control curve, 23 for the staurosporine curve, 31 for the DRB curve, and 17 for the FCCP + 2DG curve.

Table 1 Time for 50% recovery of fluorescence

Treatment	$t_{1/2}$ (s)	Mobile population (%)
Control	18.7 ± 5.7 ($n = 68$)	94.4 ± 7.7
Early G1	15.4 ± 1.7 ($n = 10$)	96.9 ± 3.8
Δ C-H1.1	$0.49 \pm 0.11^*$ ($n = 25$)	101.8 ± 4.3
Staurosporine	$42.8 \pm 18.8^*$ ($n = 46$)	91.7 ± 9.7
DRB	$40.4 \pm 20.8^*$ ($n = 62$)	93.0 ± 9.5
FCCP + 2DG	$61.6 \pm 24.0^*$ ($n = 34$)	$81.6 \pm 11.1^*$

The mean time for 50% recovery of fluorescence and the per cent mobile population during the course of the experiment was determined. For these data, more dense and less dense chromatin values were pooled. The statistical significance of the difference in recovery times relative to controls was determined by the Student's *t*-test.

*Probability that the means are equal is less than 0.0001.

energy dependence for histone H1 exchange leads us to conclude that phosphorylation, but not necessarily that of H1, is the primary modulator of histone H1 mobility and that ATP-dependent chromatin remodelling complexes make a negligible contribution to this process. Direct phosphorylation of histone H1 has been shown to alter its association with chromatin²³. Interphase phosphorylation of histone H1, in contrast to mitotic hyperphosphorylation, is correlated with the transcriptionally active state of chromatin^{24,25}, and is increased in some transformed cell lines^{16,26}. In addition, phenotype analysis in *Tetrahymena* indicates that phosphorylated histone H1 mimics H1 knockouts^{27,28}. Thus, it is possible that phosphorylation of H1 may reduce its binding affinity for chromatin *in vivo* and thereby facilitate transcriptional activation. A definitive proof of this hypothesis will require site-directed mutagenesis of the phosphorylation sites. Regardless of how H1 is displaced from chromatin, its lability provides a means to facilitate chromatin remodelling and changes in gene expression that occur as the cell responds to external signals or progresses through the cell cycle. □

Methods

Cell lines

To construct the histone H1.1–GFP fusion, we used polymerase chain reaction (PCR) amplification of the histone H1.1 with the following primers for the full-length protein: 5' primer (GGGGATCCATGG CTGAACAGTGCCT), 3' primer (AAACCGGTTTCTTGGGTGCCGCTT); HeLa genomic DNA was used as the template²⁹. Amplified H1.1 was restricted with *Bam*H1 and *Age*I, and ligated into the similarly restricted plasmid pEGFP-N1 (Clontech). Transfection of SK-N-SH cells with H1.1–GFP was achieved with LipofectAMINE reagent (Gibco BRL), and stable clones were selected with G418. Human multiple myeloma 8226 cells and Raji human plasma cells were stably transfected with the same H1.1–GFP (kindly donated by C. Maxwell, Univ. Alberta). We cultured all cells as described³⁰. Cells were grown on sterile, glass coverslips for both live cell imaging and indirect immunofluorescence.

Chromatin and protein preparation

Chromatin was prepared according to published procedures⁸ and loaded onto individual hydroxylapatite columns (Bio-Rad Econo-Pac CHT-II Cartridge). Techniques were carried out as outlined in the Bio-Rad Econo-Pac Instruction Manual. Eluted samples were frozen at –80 °C, lyophilized and resuspended in SDS–polyacrylamide gel electrophoresis sample buffer and separated on a 10% polyacrylamide gel. The identity of H1.1–GFP was confirmed by immunoblotting. We transferred proteins to nitrocellulose membrane using a Hoefer miniVE blot module. All protein analysis was done by western blot using the ECL+Plus protocol (Amersham Pharmacia). Total histone H1 primary antibody³⁰ was used at a 1:1,000 dilution; anti-rabbit HRP at 1:10,000 dilution was used as the secondary antibody.

Live cell microscopy and FRAP experiments

A laser-scanning confocal microscope (Zeiss LSM 510) with a 15-mA argon laser was used to perform all photobleaching experiments using either a 40 × 1.3 N.A. or a 25 × 0.8 N.A. objective. DIC/Normarski images were collected to monitor cell viability. We performed FRAP experiments by exposing defined regions of cells to 100% laser intensity for typically 10–20 iterations. Imaging was typically performed at 1% laser intensity. The interval between image scans varied depending on the duration of recovery in an initial pilot experiment. The interval between scans was set so that between 75 and 90 image scans were required in total for the experiment. Recovery was considered complete when the intensity of the photobleached region stabilized (that is, the curve flattened). For quantitative analysis, fluorescence intensity was measured at each time point for (1) the photobleached region, (2) the entire cell nucleus and (3) extracellular background intensity using Metamorph 4.0 (Universal Imaging) software. Background was subtracted from each data point. To determine the per cent recovery and account for the small amount of fluorescence loss owing to repeated scanning during recovery, the measured whole-nucleus fluorescence was used to normalize each data point to the fluorescence intensity of the nucleus obtained in the first image acquisition after photobleaching. The mobile population (per cent recovery) was determined by dividing the normalized photobleached region at maximal recovery by the corrected (initial photobleached region intensity multiplied by total nuclear fluorescence immediately after photobleaching divided by total nuclear fluorescence immediately before photobleaching) fluorescence intensity of the photobleached region before photobleaching. Because of the contribution of electronic noise in the detector system, some measured recoveries were observed that were greater than 100%.

Phosphorylation analysis

We treated cells with either 75 mg ml^{–1} DRB for 4 h, 1 μM staurosporine (2 h) or 50 μM FCCP with 200 mM 2DG for 4 h. The cell recovery was followed for up to 30 min in FRAP experiments to monitor histone H1.1–GFP movement.

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Dynamic binding of histone H1 to chromatin in living cells

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The linker histone H1 is believed to be involved in chromatin organization by stabilizing higher-order chromatin structure^{1–3}. Histone H1 is generally viewed as a repressor of transcription as it prevents the access of transcription factors and chromatin remodelling complexes to DNA^{4–6}. Determining the binding properties of histone H1 to chromatin *in vivo* is central to understanding how it exerts these functions. We have used photobleaching techniques to measure the dynamic binding of histone H1–GFP to unperturbed chromatin in living cells. Here we show that almost the entire population of H1–GFP is bound to chromatin at any one time; however, H1–GFP is exchanged continuously between chromatin regions. The residence time of H1–GFP on chromatin between exchange events is several minutes in both euchromatin and heterochromatin. In addition to the mobile fraction, we detected a kinetically distinct, less mobile fraction. After hyperacetylation of core histones, the residence time of H1–GFP is reduced, suggesting a higher rate of exchange upon chromatin remodelling. These results support a model in which

linker histones bind dynamically to chromatin in a stop-and-go mode.

To visualize the linker histone H1 in living cells, we fused the coding region of the green fluorescent protein (GFP) to that of mouse histone variants H1⁰ or H1c, placed under the control of a zinc-inducible promoter. We established stable cell lines in mouse BALB/c 3T3 fibroblasts as described⁷. All experiments were done in exponentially growing cells and in the absence of exogenous Zn²⁺, using only the basal activity of the promoter to prevent alterations of chromatin structure, as reported in density-arrested cells upon high overexpression of H1 variants^{7,8}. On the basis of high performance liquid chromatography (HPLC) profiling, we estimate that the cell lines overexpress less than 5% H1–GFP in addition to endogenous H1 (Fig. 1a).

By several criteria, the GFP fusion proteins behaved identically to endogenous H1 variants. Both H1⁰–GFP and H1c–GFP proteins were released from nuclei upon micrococcal nuclease digestion with kinetics identical to that of the respective unmodified endogenous protein, suggesting proper positioning of the fusion proteins on DNA (Fig. 1b)⁷. The salt-extraction properties of the H1–GFP fusion proteins were indistinguishable from those of their endogenous counterparts (Fig. 1c). Like endogenous H1, the entire pool of both H1–GFP variants was associated with chromosomes in mitotic cells, and expression of the fusion proteins had no effect on cell-cycle behaviour or cell proliferation (data not shown). In cells stably overexpressing H1⁰–GFP or H1c–GFP, the fusion protein was found diffusely distributed throughout the nucleoplasm and concentrated in 20–40 nuclear foci (Fig. 1d). The foci colocalized in paraformaldehyde-fixed cells with the heterochromatin marker protein HP1 and Hoechst 33342, an indicator of heterochromatin regions in murine cells (Fig. 1d, top)⁹. The presence of H1c–GFP in heterochromatin in living cells was confirmed by colocalization of the GFP fusions with Hoechst 33342 (Fig. 1d, bottom). Consistent

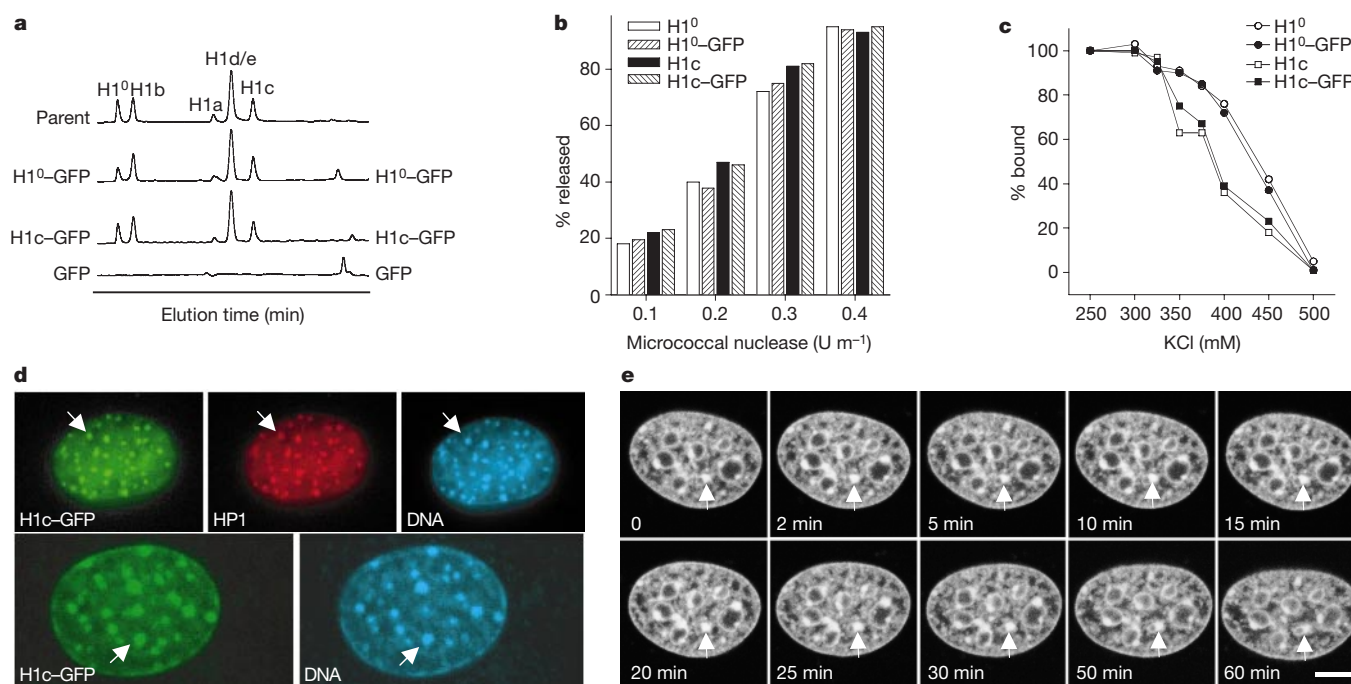


Figure 1 Properties of H1–GFP proteins. **a**, HPLC profile of H1–GFP cell lines. Total histones were extracted from either parental BALB/c 3T3 cells or a cell line stably expressing H1⁰–GFP or H1c–GFP and separated by HPLC. Recombinant GFP was used as a control. **b**, Micrococcal nuclease sensitivity. The amount of H1 released on MNase digestion was determined. **c**, Salt-extraction properties. The amount of native H1 released on extraction with increasing concentrations of KCl was determined. **d**, Localization of histone H1–GFP. Top, in fixed BALB/c 3T3 cells, stably expressed H1c–GFP colocalizes

in nuclear foci with the heterochromatin marker HP1 and Hoechst 33342 (arrows). Bottom, identical colocalization of H1c–GFP with Hoechst 33342 in heterochromatic regions of living cells (arrows). **e**, Time-lapse microscopy of H1c–GFP expressing Balb/c 3T3 cells. Images were taken every 30 s and selected images are shown. Only restricted local motion of chromatin was observed over extended observation periods (arrows). Scale bar, 3.4 μm. For experimental details see Supplementary Information.

with reports on restricted mobility of chromatin regions *in vivo*¹⁰, time-lapse microscopy experiments revealed no significant positional movements of H1c-GFP-positive foci over extended observation periods (Fig. 1e). Identical results were observed for H1⁰-GFP (data not shown).

We have previously used photobleaching techniques to show that many proteins are highly mobile in the mammalian cell nucleus¹¹. Here we applied fluorescence recovery after photobleaching (FRAP) to probe the dynamic properties of histone H1-GFP in unperturbed chromatin of living cells. After bleaching an area in the nucleus of a cell expressing either H1c-GFP or H1⁰-GFP, we recorded the recovery of fluorescence signal in the bleached area by time-lapse imaging¹¹ (Fig. 2a). As a standard we used GFP, which moves relatively freely in the nucleus and whose fluorescence signal recovers to $98 \pm 3\%$ of the pre-bleach value in less than 1 s (refs 11, 12; Fig. 2b). In contrast, H1c-GFP and H1⁰-GFP recovery was less than $1 \pm 1\%$ during the first second, less than $3 \pm 1\%$ in the first 5 s and less than $15 \pm 3\%$ during the first 20 s after bleaching (Fig. 2a, b). These recovery kinetics are significantly slower than those reported for several nuclear proteins including the chromatin-binding proteins HMG-17 (ref. 11) and HMG-14 (Fig. 2b, $P < 0.001$). These results were confirmed by fluorescence loss in photobleaching (FLIP) analysis¹¹ (see Supplementary Information). The reduced mobility of H1-GFP was the consequence of H1-GFP binding to chromatin, as mutants lacking either the globular domain (ΔG) or the carboxy-terminal domain (ΔC), both of which are involved in DNA binding, showed a significantly more rapid recovery, and their recovery level in 15 s was similar ($97 \pm 2\%$) to that of GFP alone (Fig. 2c). We conclude that in the nucleus of a living cell most H1-GFP molecules at any given time are bound to chromatin.

We characterized the dynamics of H1-GFP exchange in unperturbed chromatin by FRAP experiments using extended recovery periods. Upon bleaching a small nuclear area, slow recovery of the fluorescence signal was observed and recovery reached a plateau in 200–250 s (Fig. 3). This observation shows directly that H1-GFP is continuously exchanged between chromatin regions in the cell

nucleus (Fig. 3). For these experiments, we defined heterochromatin on the basis of morphological criteria as areas strongly labelled with H1-GFP; euchromatin was defined morphologically as areas weakly or not labelled with H1-GFP. Recovery kinetics were similar regardless of whether the bleached area was in an euchromatic or heterochromatic region (Fig. 3a–d). In both euchromatin and heterochromatin not all of the fluorescence signal was recovered (Fig. 3c, d). The recovery levels were $90.7 \pm 4.2\%$ and $74.4 \pm 2.6\%$ for H1c-GFP, and $89.6 \pm 3.8\%$ and $76.4 \pm 2.7\%$ for H1⁰-GFP, in euchromatin and heterochromatin, respectively (Fig. 3c, d). These values were significantly different ($P < 0.001$ in heterochromatin; $P < 0.005$ in euchromatin) from the recovery value for GFP alone ($98 \pm 3\%$).

This behaviour indicates the presence of two distinct kinetic pools of H1-GFP in the nucleus: a large mobile pool, which represents the continuously exchanging molecules and is responsible for the fluorescence signal recovery; and a smaller, less mobile pool, which does not contribute to the recovery. This less mobile fraction was significantly larger in heterochromatin ($\sim 25\%$) than in euchromatin ($\sim 10\%$), suggesting the presence of more statically bound H1-GFP molecules in heterochromatin than in euchromatin (Fig. 3e). This less mobile fraction might be distinct biochemically or the stronger binding might be due to properties of chromatin. We also cannot exclude the possibility that the less mobile fraction in 'euchromatin' originates from H1-GFP stably associated with interspersed heterochromatin. Because most H1-GFP molecules are bound to chromatin at any given time (Fig. 2b), H1-GFP molecules must first be released from the DNA to which they are bound at the time of bleaching to be able to move into the bleached area. Therefore, the residence time of the mobile fraction can be estimated from the recovery graphs as the time point when the recovery plateau is reached. For both histone variants in euchromatin or heterochromatin, this time was between 220 and 250 s (Fig. 3c, d, f). Our observations show that histone H1-GFP exists in at least two kinetically distinct fractions in the nucleus and that most H1-GFP molecules reside on chromatin in living cells for ~ 220 s before dissociating and rapidly re-binding to an available binding site.

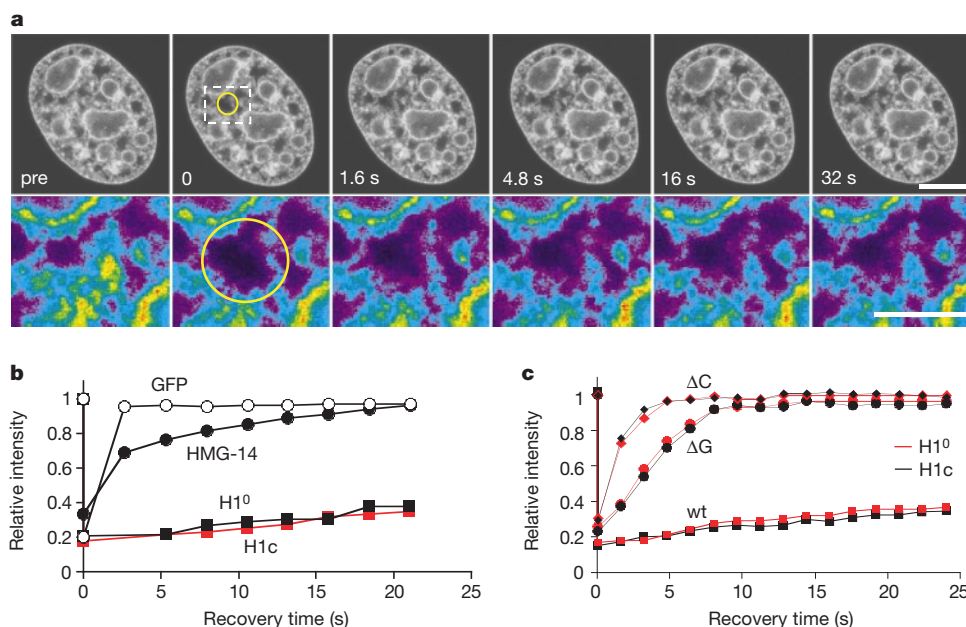


Figure 2 FRAP analysis of H1-GFP. **a**, A cell expressing H1c-GFP was imaged before and during recovery after bleaching of a nucleoplasmic area. Images were taken at the indicated times after the bleach pulse (yellow circle). The indicated area is shown enlarged in pseudocolour in the lower panel. Scale bars: 2.6 μm (top); 1.2 μm (bottom). **b**, Quantitative analysis of FRAP recovery of wild-type H1-GFP variants. The absence of

rapid recovery demonstrates the absence of a freely mobile pool of H1-GFP variants. **c**, FRAP analysis of H1-GFP variants lacking the globular domain (ΔG) or the C-terminal domain (ΔC) and wild-type (wt) H1-GFP. Mutant recovery was significantly faster than that of wild-type H1-GFP. Values represent means $\pm$ s.d. from at least 10 cells from 3 experiments.

Core histone acetylation has been implicated in changes in chromatin structure that result in the opening of chromatin and increased accessibility of DNA to remodelling factors^{6,13}. The effect of core histone hyperacetylation on the binding of histone H1 is not clear^{14–17}. Treatment of Balb/c 3T3 cells with the histone deacetylase inhibitor trichostatin A (TSA) results in the marked increase in the levels of acetylation of all four core histones⁸. In FRAP experiments of TSA-treated cells, the recovery of H1⁰-GFP and H1c-GFP was complete in 100–150 s in both euchromatin and heterochromatin; this was significantly faster than the recovery time of 220–250 s in untreated cells (Fig. 4a–d; $P < 0.001$). The increase in recovery rate

translates into a shorter residence time on TSA treatment (Fig. 4e). Although recovery levels in the euchromatin of TSA-treated cells were similar to those of control cells ($\sim 90\%$), the recovery level in heterochromatin was increased from $\sim 75\%$ in control cells to $87 \pm 4\%$ for H1c-GFP ($P < 0.001$) and $92 \pm 4\%$ for H1⁰-GFP ($P < 0.005$; Fig. 4c, d, f). This difference indicated that the size of the less mobile fraction of H1-GFP variants in heterochromatin was reduced from 25% in control cells to $13 \pm 4\%$ for H1⁰-GFP and to $8 \pm 4\%$ for H1c-GFP on TSA treatment ($P < 0.001$ for H1⁰-GFP; $P < 0.005$ for H1c-GFP) (Fig. 4f). The increased exchange rate was not a consequence of the transcriptional activity of the cell, as

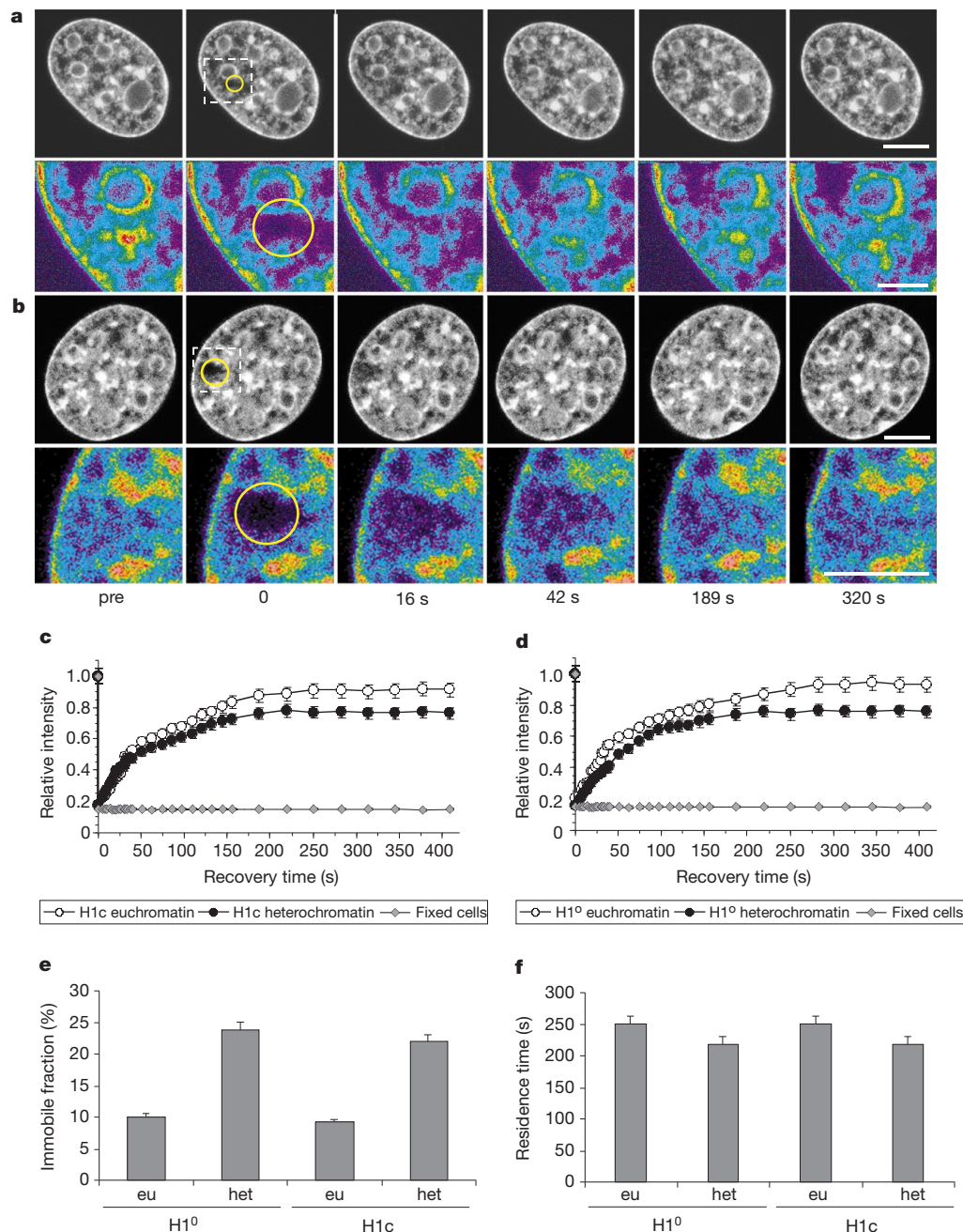


Figure 3 FRAP on H1-GFP in heterochromatin or euchromatin. **a, b**, Cells expressing H1c-GFP were imaged before and during recovery after bleaching of either a heterochromatic (**a**) or an euchromatic (**b**) area (yellow circle). Images were taken at the indicated times after end of the bleach pulse. The indicated area is enlarged in pseudocolour in the bottom panels. Scale bars: 3.2 μm (top); 1 μm (bottom). **c, d**, Quantitative analysis of FRAP experiments after bleaching of H1c-GFP (**c**) or

H1⁰-GFP (**d**) in either an euchromatic (open circles) or heterochromatic (filled circles) region of the nucleus. **e, f**, Immobile fraction (**e**) and residence time (**f**) of H1⁰-GFP or H1c-GFP in heterochromatin or euchromatin. The residence time is similar in heterochromatin and euchromatin. The immobile fraction is larger in heterochromatin than in euchromatin. Values represent means $\pm$ s.d. from at least 10 cells from 3 experiments.

inhibition of transcription alone by α -amanitin did not have any effect on the recovery rate (data not shown). These observations suggest that hyperacetylation increases the exchange rate of histone H1-GFP from chromatin of living cells.

We have used photobleaching techniques to characterize the binding dynamics of the linker histone H1-GFP to unperturbed chromatin *in vivo*. To our knowledge this is the first measurement of the residence time of a DNA-binding protein in chromatin of living cells. We find that at any given time, most histone H1-GFP molecules are bound to chromatin. However, most H1-GFP molecules are continuously exchanged between chromatin regions. These observations lead to the conclusion that most H1-GFP molecules bind chromatin by a 'stop-and-go' mechanism: a histone H1-GFP molecule resides on chromatin for ~ 220 s. The molecule then dissociates and rapidly binds to another binding site. On the basis of the mobility properties of other nuclear proteins, we speculate that the intermediate in this exchange is freely mobile and moves by diffusion in the nucleoplasm¹¹.

Transfer of H1-GFP between chromatin segments has been suggested by *in vitro* salt-extraction studies and cell fusion experiments of cells micro-injected with labelled H1 (refs 18–21).

However, those earlier studies could not determine the residence time of the mobile fraction of histone H1, nor did they reveal the presence of kinetically distinct pools of H1. At present, we do not know the functional or physiological significance of the two fractions, and it is not clear how these two fractions differ at the molecular level. An interesting possibility is that protein phosphorylation, which alters the DNA interaction properties of H1 (refs 22, 23), can interconvert the two pools. We also find evidence for increased exchange of H1-GFP between chromatin regions on hyperacetylation. Increased core histone acetylation is a hallmark of transcriptionally active chromatin, and results, in many cases, from the action of chromatin remodelling activities^{6,24,25}. On treatment of cells with TSA, we observed an increased exchange rate of the mobile fraction of H1-GFP, suggesting a 'looser' binding of histone H1-GFP to chromatin. These results extend into living-cells biochemical studies showing that fractionated 'active' chromatin is relatively depleted in H1 and enriched in acetylated core histones^{26,27}. We propose that the dynamic nature of binding is an essential feature of linker histones in their functions as regulators of chromatin remodelling and chromatin structure *in vivo*. $\square$

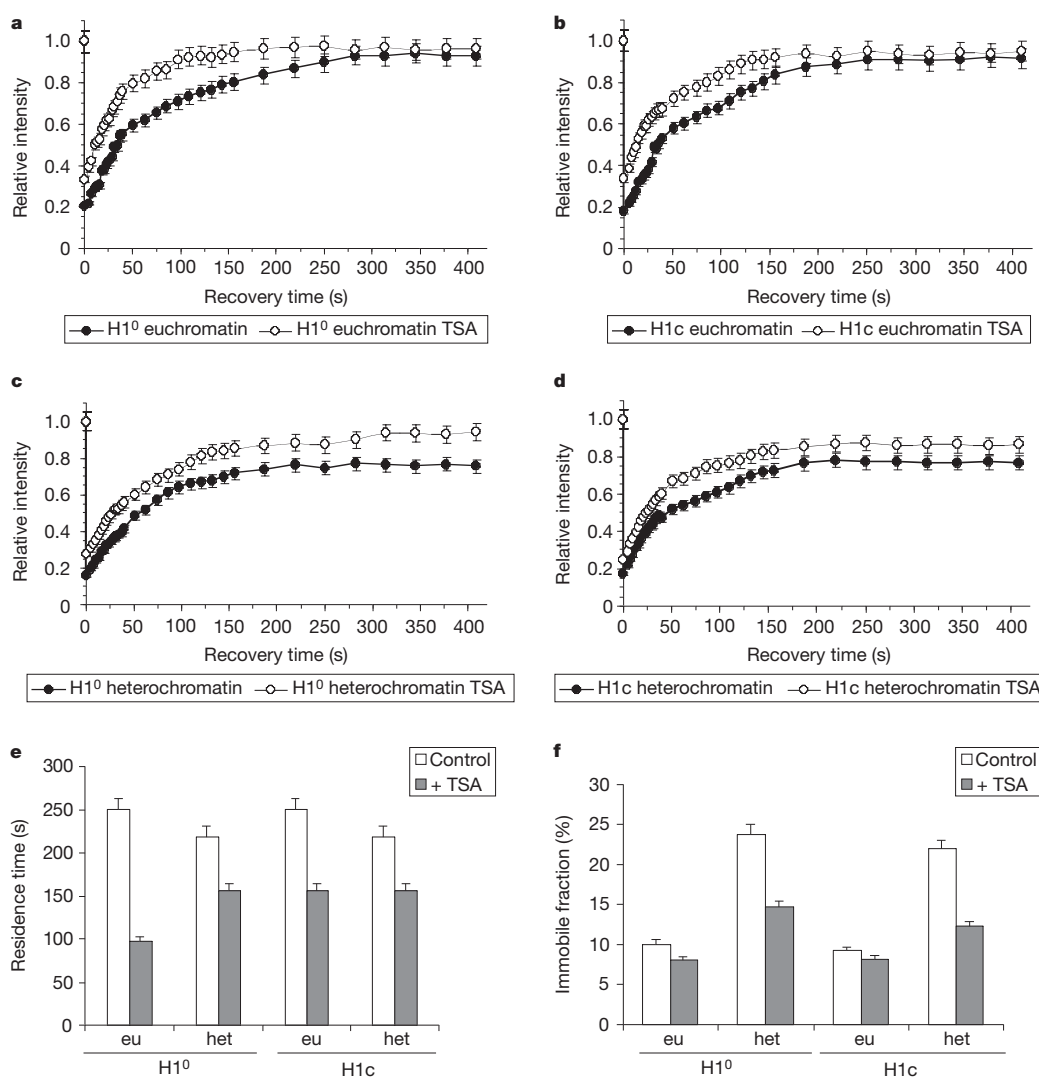


Figure 4 FRAP on H1-GFP in heterochromatin or euchromatin after TSA treatment. Cells were treated with 50 ng ml⁻¹ TSA for 2 h before FRAP analysis. **a-d**, Quantitative analysis of FRAP experiments after bleaching of either variant in heterochromatin (open circles) or euchromatin (filled circles). **e, f**, Residence time (**e**) and immobile fractions (**f**) of H1⁰-GFP or H1c-GFP in heterochromatin or euchromatin after TSA treatment as compared with

control cells. The residence time of H1-GFP in euchromatin and heterochromatin was reduced upon TSA treatment. The immobile fraction of H1-GFP in heterochromatin, but not euchromatin, was reduced. Values represent means $\pm$ s.d. from at least 10 cells from 3 experiments.

Methods

Constructs and cell lines

The coding sequence for enhanced GFP was excised from pEGFP-C1 (Clontech) and was inserted after the last lysine residue of the H1 variants in pMTH1^{cneo} or pMTH1^oneo (ref. 7). An additional alanine residue was inserted between GFP and the H1 variant during the cloning process. Deletion mutants lacking the C-terminal domain (Δ C) or the central globular domain (Δ G) were generated by standard subcloning methods (see Supplementary Information). H1^o Δ C contained residues 1–91; H1c Δ C, 1–104; H1^o Δ G, 1–20 and 77–193; H1c Δ G, 1–35 and 142–211. Stable cell lines expressing H1–GFP fusion proteins were generated from BALB/c 3T3 fibroblasts and grown as described⁷. More than 15 cell lines for each variant were generated. For details see Supplementary Information.

HPLC profiling, nuclease digestion and salt extraction

Expression levels of H1–GFP fusion proteins were determined by extraction of total chromatin-bound histones and quantitative separation by HPLC as described²⁸. Release of H1–GFP proteins on micrococcal nuclease (MNase) digestion of nuclei was determined by fluorimetry of MNase-soluble material as described⁷. Release of H1 proteins on MNase digestion of the same sample was determined by quantitative HPLC of MNase-insoluble material as described⁷. To measure the salt extraction of H1 variants, crude nuclei were extracted with high salt buffer containing increasing concentrations of KCl. Total histones in the pelleted material were analysed by quantitative HPLC as described²⁸. For details see Supplementary Information.

Indirect fluorescence and *in vivo* microscopy, and FRAP

Indirect immunofluorescence microscopy was carried out as described²⁹, using fixation in 2% buffered paraformaldehyde in PBS for 15 min at room temperature. A rabbit polyclonal antibody against HP1 (kindly provided by E. Minc, Institute Pasteur, Paris) was used at 1:500 in PBS⁹. Hoechst stain 33342 was used at 500 ng ml^{−1} for 10 min at room temperature. Cells were mounted using Vectashield and observed on a Nikon E800 microscope using a $\times 60$ Planapo Nikon objective (N.A. 1.4) and a Photometrics MicroMax cooled CCD camera (1,300 $\times$ 1,030 array, 6.7 μ m pixel size, 5 MHz, image pixel size 80 nm). Images were generated and analysed in Metamorph (Universal Imaging). For live-cell microscopy, we grew cells to 50% confluence in Nalgene LabTek II chambers and carried out microscopy with a Leica TCS-SP confocal microscope using the 488-nm laser line of an argon laser (20 mW nominal output, beam width at specimen 0.2 μ m, detection 500–575 nm using a photon multiplier tube with pinhole setting at 1, image pixel size 47 nm). All experiments were done at 37°C.

Experimental details of FRAP have been described¹¹. Five single imaging scans were acquired, followed by a single bleach pulse of 200–500 ms using a spot of $\sim 1 \mu$ m in diameter without scanning. Single-section images were then collected at 1-s intervals (15 images), followed by 10-s intervals (10 images) and then by 30-s intervals (8 images). For imaging, the laser power was attenuated to 1% of the bleach intensity. FRAP recovery curves were generated from background-subtracted images. The total cellular fluorescence was determined for each image and compared with the initial total fluorescence to account for the amount of signal lost during the bleach pulse and during imaging as described¹¹. Relative recoveries were determined as described¹¹. In FRAP experiments, typically $\sim 10\%$ of total fluorescence was lost during the bleach pulse and less than 5% of fluorescence was lost during the entire imaging phase. The level of autofluorescence in cells not expressing the fusion proteins was 1% or less of that of expressing cells. The standard Student's *t*-test was used to determine the statistical significance of results. All quantitative values represent averages from at least 10 cells from 3 independent experiments.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome

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Introns are removed from nuclear messenger RNA precursors through two sequential phospho-transesterification reactions in a dynamic RNA–protein complex called the spliceosome^{1,2}. But whether splicing is catalysed by small nuclear RNAs^{3,4} in the spliceosome is unresolved. As the spliceosome is a metalloenzyme^{5–7}, it is important to determine whether snRNAs coordinate catalytic metals. Here we show that yeast U6 snRNA coordinates a metal ion that is required for the catalytic activity of the spliceosome. With Mg²⁺, U6 snRNA with a sulphur substitution for the pro-R_p or pro-S_p non-bridging phosphoryl oxygen of nucleotide U₈₀ reconstitutes a fully assembled yet catalytically inactive spliceosome. Adding a thiophilic ion such as Mn²⁺ allows the first transesterification reaction to occur in the U6/sU₈₀(S_p)- but not the U6/sU₈₀(R_p)-reconstituted spliceosome. Mg²⁺ competitively inhibits the Mn²⁺-rescued reaction, indicating that the metal-binding site at U6/U₈₀ exists in the wild-type spliceosome and that the site changes its metal requirement for activity in the S_p spliceosome. Thus, U6 snRNA contributes to pre-messenger

RNA splicing through metal-ion coordination, which is consistent with RNA catalysis by the spliceosome.

The spliceosome forms by incorporating small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins onto pre-mRNA, and it attains its catalytic conformation by undergoing a series of protein and RNA rearrangements^{1–3,8}. Much evidence indicates that splicing is catalysed by the snRNAs in the spliceosome, although there is no definitive proof^{3,4}. Among the five spliceosomal snRNAs (U1, U2, U4, U5 and U6), U6 is most widely assumed to be involved in catalysis because it is highly conserved through evolution and very sensitive to chemical changes. In addition, U6 forms intramolecular and intermolecular helices that are analogous to autocatalytic group II introns³. If U6 snRNA were directly involved in catalysis, it might coordinate catalytic metals, because the spliceosome, like most RNA enzymes⁹, uses divalent metal ions in catalysis^{6,7}. If a phosphoryl oxygen/Mg²⁺ interaction is crucial for function, a sulphur substitution for that oxygen would cause a switch in metal specificity from Mg²⁺ to a thiophilic ion such as Mn²⁺ (ref. 10). Several (R_P)-phosphorothioate substitutions in yeast or nematode U6 snRNA fail to reconstitute active spliceosomes with Mg²⁺ (refs 11, 12). However, Mn²⁺ does not rescue these phosphorothioates, and it is unclear whether any of the oxygens coordinate metal^{13,14}.

We tested synthetic U6 snRNA containing a single phosphorothioate (as a mixture of R_P and S_P diastereomers) in reconstituting Mg²⁺-dependent splicing in a U6-depleted yeast extract^{11,15}. Many phosphorothioate-containing U6 RNAs reconstituted splicing well (our own unpublished data), except U6 with a sulphur substitution at U₈₀ (Fig. 1, lane 5). Pure sU₈₀(R_P) diastereomer completely failed to reconstitute splicing (Fig. 1, lane 6)¹¹. Notably, splicing also did not occur in the extract reconstituted with U6/sU₈₀(S_P) (Fig. 1, lane 7), suggesting that both 5' non-bridging oxygens of U₈₀ are important for splicing.

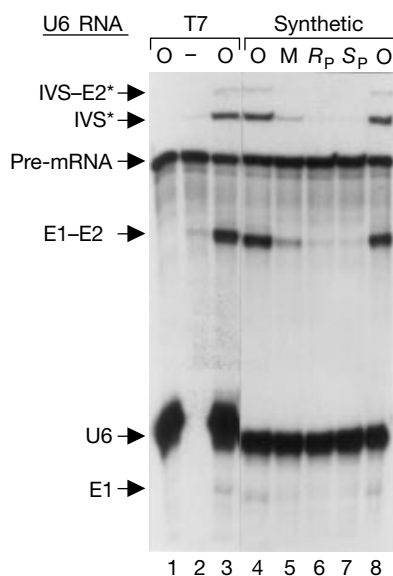


Figure 1 U6 snRNA with a phosphorothioate substitution at U₈₀ fails to reconstitute splicing. U6-depleted *prp2* mutant extracts were reconstituted with T7 polymerase-transcribed, unmodified U6 (lanes 1 and 3) or ligated, synthetic U6 (lanes 4–8) in the absence (lane 1) or presence (lanes 2–8) of wild-type PRP2 protein. No U6 was added in lane 2 (–). Both input pre-mRNA and U6 RNA were labelled with ³²P and were analysed by denaturing gels and autoradiography. Synthetic U6 used: O, no phosphorothioate substitution (lane 4); M, unpurified R_P/S_P phosphorothioate mixture with some unsulphurized RNA (lane 5); and the R_P diastereomer (lane 6), S_P (lane 7) and the unsulphurized RNA (lane 8) purified from the mixture. The intron–exon 2 lariat (IVS–E2*) and exon 1 (E1) are products from the first transesterification reaction, and the intron lariat (IVS*) and ligated exon 1/exon 2 (E1–E2) are from the second reaction.

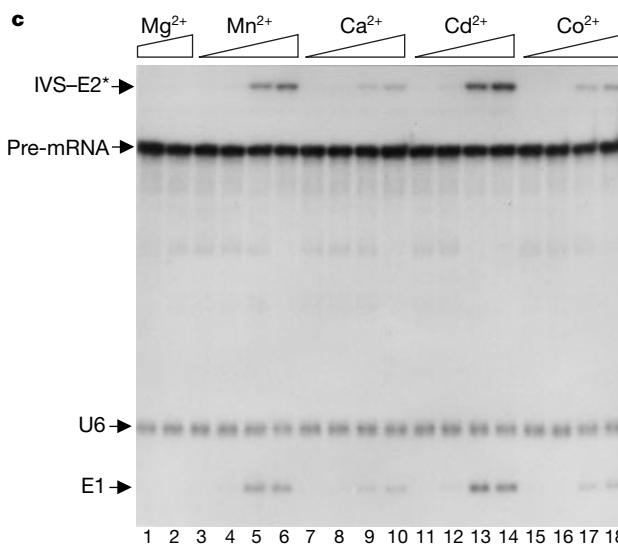
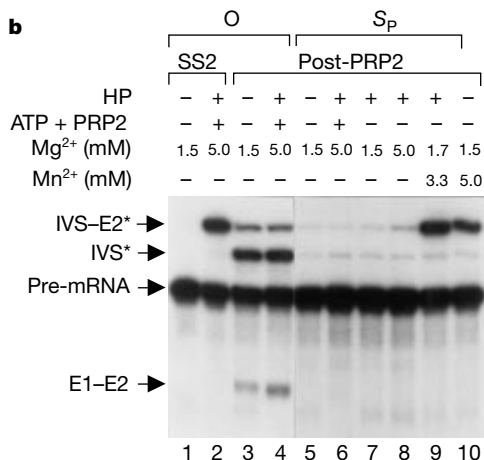
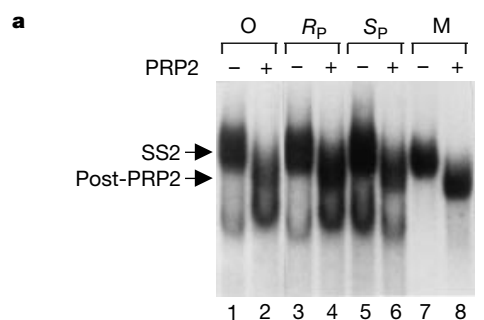


Figure 2 Spliceosome maturation is not affected by phosphorothioate substitution at U₈₀ of U6 but splicing only occurs in the S_P diastereomer with a thiophilic metal ion. **a**, U6/sU₈₀ forms mature spliceosome. Extracts were reconstituted with unmodified U6 (lanes 1 and 2) or U6/sU₈₀ (R_P, lanes 3–4; S_P, lanes 5–6), in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of PRP2. The reaction mixture was sedimented through a glycerol gradient and the fraction containing the spliceosome was analysed on a native gel. The pre-catalytic and post-PRP2 spliceosomal markers (M; lanes 7 and 8) were generated as described¹⁶. **b**, The first transesterification reaction occurs in U6/sU₈₀(S_P)-containing post-PRP2 spliceosome in the presence of Mn²⁺. Spliceosomes isolated from glycerol gradients were incubated in buffer containing combinations of Mg²⁺, Mn²⁺, ATP, PRP2 and HP (a protein factor that facilitates splicing in purified spliceosomes¹⁶). **c**, Other thiophilic divalent metal ions can also rescue the transesterification reaction. The post-PRP2 U6/sU₈₀(S_P) spliceosome was incubated with metal ions without the addition of ATP, PRP2 or HP. Mg²⁺ was added as the sole divalent metal ion in lanes 1 (2.5 mM) and 2 (5 mM). The concentrations (mM) of the thiophilic metal (Mn²⁺, Ca²⁺, Cd²⁺ or Co²⁺) in each set were 0.02, 0.1, 0.5 and 2.5. Mg²⁺ was added to lanes 3–18 to make the total concentration of divalent metal ions 5 mM.

We investigated whether U6/sU₈₀ snRNA blocked spliceosome maturation or Mg²⁺-dependent transesterification. The reactions were carried out with or without the yeast PRP2 ATPase, which is required for the final ATP-dependent spliceosomal rearrangements before the first transesterification reaction¹⁶. Reconstituted spliceosomes were isolated from glycerol gradients and analysed on a non-denaturing gel (Fig. 2a). In the absence of PRP2, unmodified U6, U6/sU₈₀(R_P) and U6/sU₈₀(S_P) reconstituted a pre-catalytic SS2 spliceosome¹⁶ (Fig. 2a, lanes 1, 3 and 5), which was equivalent to mammalian spliceosome B2 (ref. 1). In the presence of PRP2, the post-PRP2 spliceosomes were formed (Fig. 2a, lanes 2, 4 and 6),

which were equivalent to mammalian spliceosomes C1 and C2 (ref. 1). These spliceosome complexes were not detected in U6-depleted extracts without reconstitution (data not shown). Thus, both of the U6/sU₈₀ RNAs support spliceosome maturation but not catalysis.

We tested whether phosphorothioate substitutions at U₈₀ switch the metal requirement for catalysis by incubating the purified post-PRP2 spliceosomes with Mg²⁺ or Mn²⁺ (Fig. 2b). Splicing did not occur in the sU₈₀(R_P) spliceosome regardless of metal used (data not shown). No splicing occurred when the sU₈₀(S_P) spliceosome was incubated with Mg²⁺ as the sole divalent metal ion (Fig. 2b, lanes 5–8). However, the sU₈₀(S_P) spliceosome efficiently carried out the first transesterification reaction in the presence of Mn²⁺ (Fig. 2b, lanes 9 and 10). This reaction did not require additional ATP, PRP2 or the HP splicing factor, confirming that this spliceosome had been fully activated¹⁶. Other thiophilic divalent metal ions also rescued the sU₈₀(S_P) spliceosome; Cd²⁺ was more effective than Mn²⁺, whereas Ca²⁺ and Co²⁺ were less effective (Fig. 2c). Rescue occurred at submillimolar concentrations, indicating that there was specific metal interaction at the site of sulphur substitution¹⁷. Thus, these results showed that only the sU₈₀(S_P) and not sU₈₀(R_P) phosphorothioate caused a switch of metal specificity, and that the transesterification reaction could occur in the S_P spliceosome once thiophilic metal occupied the sulphur-substituted site.

As purified spliceosomes are missing second-step factors¹⁶, we tested the ability of Mn²⁺ to rescue the second transesterification reaction in extracts (Fig. 3a). Mn²⁺ rescued the first but not the second transesterification reaction in U6/sU₈₀(S_P)-reconstituted extracts (Fig. 3a, lanes 14–18), whereas the U6/sU₈₀(R_P)-reconstituted extract could not be rescued (lanes 8–12). This specificity of rescue indicated that desulphurization of the phosphorothioate did not occur during the reaction. The result also suggests that the pro-S_P oxygen of U₈₀ may have a different role during the second step. The pro-S_P and pro-R_P oxygens, for example, may switch roles in the two spliceosomal active sites¹⁸.

We further characterized the Mn²⁺-rescued site by testing whether Mg²⁺ could competitively inhibit the Mn²⁺-supported reaction in U6/sU₈₀(S_P). Pre-catalytic SS2 spliceosomes were reconstituted

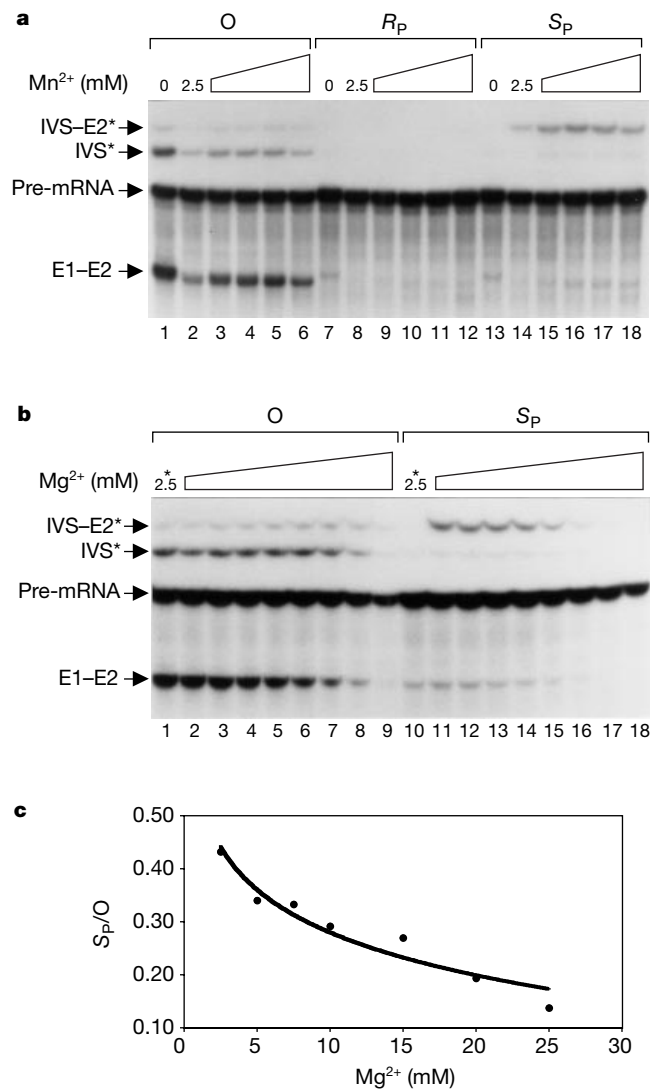


Figure 3 With U6/sU₈₀(S_P) RNA, only the first of the two transesterification reactions occurs in the presence of Mn²⁺, which is competitively inhibited by Mg²⁺. **a**, Mn²⁺ rescues the first but not the second step of splicing in U6/sU₈₀(S_P)-reconstituted reaction. Extract was reconstituted with U6 (lanes 1–6), U6/sU₈₀(R_P) (lanes 7–12), or U6/sU₈₀(S_P) (lanes 13–18) and PRP2 in the presence of Mn²⁺ and Mg²⁺. In each set of six reactions, the concentrations (mM) of Mn²⁺/Mg²⁺ were 0/2.5, 2.5/2.5, 1.25/1.25, 1.7/0.8, 2.0/0.5, 2.5/0. **b**, Mg²⁺ competitively inhibits the Mn²⁺-rescued reaction. Extract was reconstituted in the absence of PRP2 with U6 (lanes 1–9) or U6/sU₈₀(S_P) (lanes 10–18) and with Mg²⁺ (2.5 mM) as the sole divalent metal ion. Splicing was then triggered by the addition of PRP2 with 1 mM Mn²⁺ (lanes 2–9 and 11–18) and an increasing concentration (mM) of Mg²⁺ (2.5, 5.0, 7.5, 10, 15, 20, 25, 30). Reactions in lanes 1 and 2 contained 2.5 mM of Mg²⁺ and no Mn²⁺ (asterisk). **c**, The ratio of splicing efficiency between the U6/sU₈₀(S_P)- and U6-reconstituted reactions (S_P/O). Splicing efficiency in each reaction (Fig. 3b) was calculated as the ratio between the products (IVS-E2*, IVS* and E1-E2) and the total (products plus pre-mRNA).

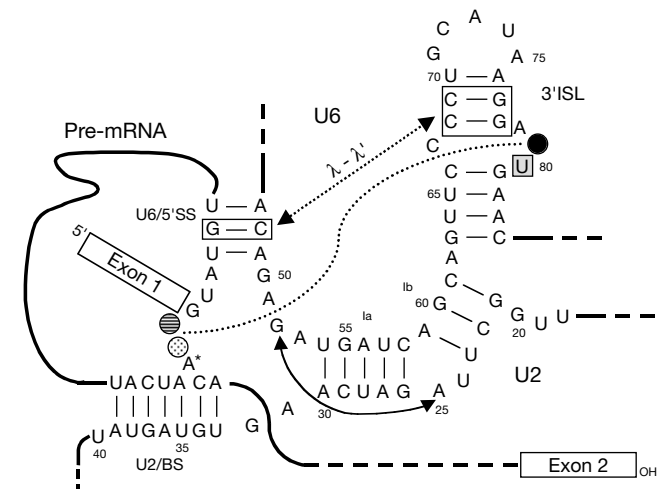


Figure 4 A representation of RNAs and metal ions in the yeast spliceosome before the first catalytic step. Intermolecular helices between U6 and U2 (la and lb), U2 and pre-mRNA at the branch site (U2/BS), U6 and pre-mRNA at the 5' splice site (U6/5'SS), as well as the 3' intramolecular stem-loop (3'ISL) in U6 are depicted^{1–3,8}. The branchpoint adenine is highlighted with a star. The U₈₀ of U6 is shaded. Three Mg²⁺ ions are shown as circles: one stabilizes the leaving oxygen at the 5' scissile phosphate⁶ (lined); one activates the attacking oxygen⁵ (dotted); and one binds to the pro-S_P oxygen of U₈₀ (filled). The tertiary interaction between G₅₂ of U6 and A₂₅ of U2 (refs 26, 27), a putative interaction between 3'ISL of U6 and the U6/5'SS helix based on the λ-λ' interaction in a group II intron²⁸, and the potential relationship between the metals are linked with a line.

stituted with unmodified U6 or U6/sU₈₀(S_P) and, without gradient purification, incubated with PRP2 and Mn²⁺ plus an increasing amount of Mg²⁺ (Fig. 3b). The ratio of splicing efficiency between the U6/sU₈₀(S_P)-reconstituted reaction and the U6-reconstituted reaction decreased as the Mg²⁺ concentration increased (Fig. 3c). Thus, Mg²⁺ could effectively occupy the Mn²⁺-rescued site in U6/sU₈₀(S_P) but it could not support splicing. This result indicates that sulphur substitution does not create a binding site for the rescuing metal ion that is not present in the wild-type reaction^{19,20}. It also indicates that the metal-binding site at U₈₀ changes its metal requirement for activity in the U6/sU₈₀(S_P)-reconstituted spliceosome.

We have identified, to our knowledge, the first snRNA site that contributes to catalysis in the spliceosome through metal-ion coordination. Ribozymes such as hammerhead^{21,22}, group I intron of *Tetrahymena*²³ and RNase P (ref. 24) have phosphoryl oxygens coordinating metal that contributes to catalysis. It will be of interest to know the exact geometry of the Mg²⁺ coordinated by the pro-S_P oxygen of U₈₀ with respect to the 5' splice site, and whether this metal ion is the one that interacts with the leaving group⁶. U₈₀ of the yeast U6 snRNA is situated at the bulge region of its 3' intramolecular stem loop (3'ISL, Fig. 4). This 3'ISL of U6 and the helix Ib of U2–U6 have been proposed to be functionally analogous to the catalytically important domain 5 of group II introns, partly on the basis of (R_P)-phosphorothioate interference analysis^{12,25}. For U₈₀ to coordinate a Mg²⁺ at or near the 5' splice site, it has to fold over to the scissile phosphate. A tertiary interaction between U2/A₂₅ and U6/G₅₂ identified in yeast²⁶ and in human²⁷ might be involved in this folding. Tertiary interactions (λ–λ') have recently been described in a group II intron between the conserved G at the fifth position of the intron and the two conserved G–C base pairs above the corresponding U₈₀ bulge in domain 5 (ref. 28). If similar interactions also occurred in the spliceosome, they could juxtapose the U₈₀ of U6 and the 5' splice site. Thus, U₈₀ could coordinate the Mg²⁺ that stabilizes the leaving group at the 5' splice junction⁶, the putative Mg²⁺ that activates the nucleophilic hydroxyl at the branchpoint⁵, or an Mg²⁺ nearby (Fig. 4). In conclusion, we have elucidated a catalytic role for the U6 snRNA in pre-mRNA splicing, and provided evidence to support the notion that the spliceosome is fundamentally an RNA enzyme. □

Methods

RNA oligonucleotides

We synthesized RNA oligonucleotides using CE (β-cyanoethyl) phosphoramidites with 2'-TBDMS (*t*-butyl-dimethylsilyl) protecting groups (Glen Research) on an ABI 394 DNA/RNA synthesizer at the Caltech Biopolymer Center. Sulphurization of a non-bridging oxygen was performed by using 3H-1,2-benzodithiol-3-one-1,1-dioxide. After synthesis, we deprotected the preparation using triethylamine trihydrofluoride, and purified the full-length oligonucleotide from a denaturing polyacrylamide gel or through a diethylaminoethyl (DEAE) column. The oligomer containing sU₈₀ was resolved by a C18 Beckman Ultrasphere ODS column on a Hewlett-Packard high-performance liquid chromatography (HPLC) into three peaks: first, the unmodified oligonucleotide; second, the R_P diastereomer; and third, the S_P diastereomer. The exact mass of the oligomers was confirmed by mass spectrometry on a Voyager Elite instrument at the Caltech Protein/Peptide Micro Analytical Laboratory (PPMAL) facility. The R_P and S_P diastereomers were verified by digesting ³²P-labelled oligomers with snake venom phosphodiesterase, which preferentially cleaves the R_P isomer²⁹.

Pre-mRNA and U6 snRNA

SP6 RNA polymerase was used to produce ³²P-labelled actin pre-mRNA¹⁶. U6 snRNA was made by using T7 RNA polymerase¹¹ or by ligating four synthetic RNA oligonucleotides^{15,30}. Four RNA oligos, U6_{1–37}, U6_{38–69}, U6_{70–83}, and U6_{84–112} were ligated to generate U6/U₈₀ RNA on a DNA template, cdU6_{19–100}. Before ligation, three RNA oligomers, U6_{38–69}, U6_{70–83}, and U6_{84–112} were pooled and phosphorylated for 60 min at 37 °C in 10 μl reaction containing 50 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 60 μM ATP, 10 pmol [γ-³²P]ATP, 10 units of RNasin and 10 units of T4 polynucleotide kinase. The phosphorylation reaction was terminated by heating at 65 °C for 20 min. The fourth RNA oligomer, U6_{1–37}, and the DNA template, cdU6_{19–100}, were then added and the mixture was heated at 90 °C for 1 min and cooled at room temperature. Ligations were performed at 30 °C for 4 h in 25 μl reaction containing 5,000 units of T4 DNA ligase, 20 units of RNasin, 4% of PEG and 1 × T4 DNA ligase buffer (New England Biolabs). Ligated RNA was isolated from a 7.5% polyacrylamide/urea gel.

Splicing and spliceosome assays

Extract preparation, *in vitro* splicing, gradient and gel analysis of spliceosomes, and spliceosome conversion assays were done as described¹⁶. U6 reconstitution was done by cleaving endogenous U6 snRNA in heat-inactivated *prp2* mutant extracts in the presence of 450 nM d1 (cdU6_{28–54}) oligodeoxyribonucleotide and 2 mM ATP¹¹. Pre-mRNA (0.4 nM), U6 RNA (4 nM), divalent metal ions in chloride form, potassium phosphate and PEG (4.8%) were added and incubated at 23 °C for 30 min to allow reconstitution and spliceosome assembly. The PRP2 protein was added and incubated for splicing at 23 °C for 15 min. ³²P-labelled RNA on gels was quantitated with a Phosphorimager.

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